ALKALOID BIOSYNTHESIS IN CULTURED TISSUES OF DUBOISIA(Dissertation_全文)

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CONTENTS

ABBREVIATIONS

BA	6-benzyladenine
DAPI	4',6-diamino-2-phenylindoledihydrochloride
EDTA	ethylenediaminetetraacetic acid
GC-MS	gas chromatography - mass spectrometry
GLC	gas-liquid chromatography
IBA	indolebutyric acid
MABA	4-methylaminobutyraldehyde
MES	2-(N-morpholino)ethanesulphonic acid
NAA	naphthaleneacetic acid
TMS-	trimethylsilyl-
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

Duboisia: botany and distribution

Genus Duboisia (family Solanaceae, tribe Salpiglossideae) comprises three species endemic to Australia: Duboisia myoporoides, D. leichhardtii and D. hopwoodii. The former two species have been of particular economic interest since large amounts of l-hyoscyamine and scopolamine, high valued spasmolytics and anesthetics, were found in their leaves. These plants, along with their sexual hybrids which accumulate high levels of the alkaloids, are now widely cultivated in Australia and Indonesia as commercial sources of these alkaloids. The habit and distribution of Duboisia species in Australia were described by Barnard (1952).

Duboisia myoporoides is a tree with lanceolate to obivate glabrous leaves 6-10 cm long, and its trunk has very corky bark. This species is distributed along the east coast of Australia, where the rainfall exceeds a monthly average of 50 mm for 11 months of the year and frost rarely occurs. In its natural state it occurs as isolated trees on the edge of forests.

Duboisia leichhardtii has smaller and narrower leaves and slightly larger flowers than D. myoporoides. The distribution of this species is limited to a small region in southeastern Australia. The general habit of this species is similar to that of D. myoporoides.

D. hopwoodii is a small shrub with narrow lanceolate leaves which are smaller than those of D. leichhardtii. It grows scattered throughout the drier regions of Australia, mostly within the 250 mm rainfall zone. It generally in-

habits sandy open country and is found as scattered individuals or in small groups. Its leaves, which contain tobacco alkaloids, were used before the coming of the white man as a chewing narcotic by the aborigines.

Alkaloids in Duboisia

The main alkaloids of Duboisia leichhardtii and D. myoporoides are hyoscyamine and scopolamine, respectively. The alkaloid contents in these Duboisia species are much higher than those found in Hyoscyamus, Scopolia, Atropa and Datura species, which have been major sources of hyoscyamine and scopolamine. The third species, D. hopwoodii, is unique in alkaloid production: this species produces considerable amounts of nicotine and nornicotine, both of which are pyridine-type alkaloids normally found in Nicotiana species. Although the characteristic alkaloid spectra of the three Duboisia species have been studied chiefly by analyses of plant leaves, both tropane and nicotine (pyridine) alkaloids were found in the roots of all three species (Kennedy 1971, Luanratana and Griffin 1982). No other plant species have been reported to produce tropane and nicotine alkaloids together. This unique alkaloid spectra has attracted the interest of chemotaxonomists. It has been proposed that the Duboisia species are a taxonomic link between the genus Nicotiana, which produces nicotine alkaloids, and the genera such as Hyoscyamus, Atropa and Datura which produce tropane alkaloids (Walker and Nowacki 1978).

Biosynthetic pathway of tropane and nicotine (pyridine) alkaloids

The pathways leading to tropane and tobacco alkaloids have been established through numerous experiments involving *in vivo* feeding of radioactive precursors (Leete 1979, 1980). The generally accepted pathway is shown in Fig. 1. The tropane alkaloids, hyoscyamine and scopolamine, share a common precursor, 4-methylaminobutyraldehyde (MABA), with tobacco alkaloids such as nicotine and nornicotine. MABA, which is derived from the amino acids ornithine or arginine, exists in equilibrium with N-methylpyrrolinium salt. When MABA is coupled with nicotinic acid, nicotine is synthesized. On the other hand, hygrine, a precursor of the tropane alkaloids, is formed from MABA and an unknown counterpart, possibly acetoacetic acid, acetoacetyl CoA, or acetonedicarboxylic acid. Hygrine is incorporated into the tropane moiety of hyoscyamine. Tropic acid is formed from phenylalanine through intramolecular rearrangement (Leete et al. 1975). Epoxidation of hyoscyamine to scopolamine occurs via 6β-hydroxyhyoscyamine (Hashimoto and Yamada 1987 a,b). The involvement of 6,7-dehydrohyoscyamine, a putative intermediate, in this epoxidation step has recently been refuted by the results of feeding experiments with [6-^{18}O]6β-hydroxyhyoscyamine (Hashimoto et al. 1987).

In this study I investigated alkaloid production in cultured tissues of *Duboisia* species. In chapter I, the relationship between organogenesis and alkaloid synthesis is described. Alkaloid production in cultured roots and synthesis of hygrine, an intermediate in the biosynthetic pathway leading to the tropane alkaloids, are also described. In chapter II, somatic hybridization between *Duboisia* species which produce both tropane and nicotine alkaloids,

and Nicotiana tabacum which produce only nicotine alkaloid, is demonstrated. Alkaloid biosynthesis in the intergeneric somatic hybrids is explored.

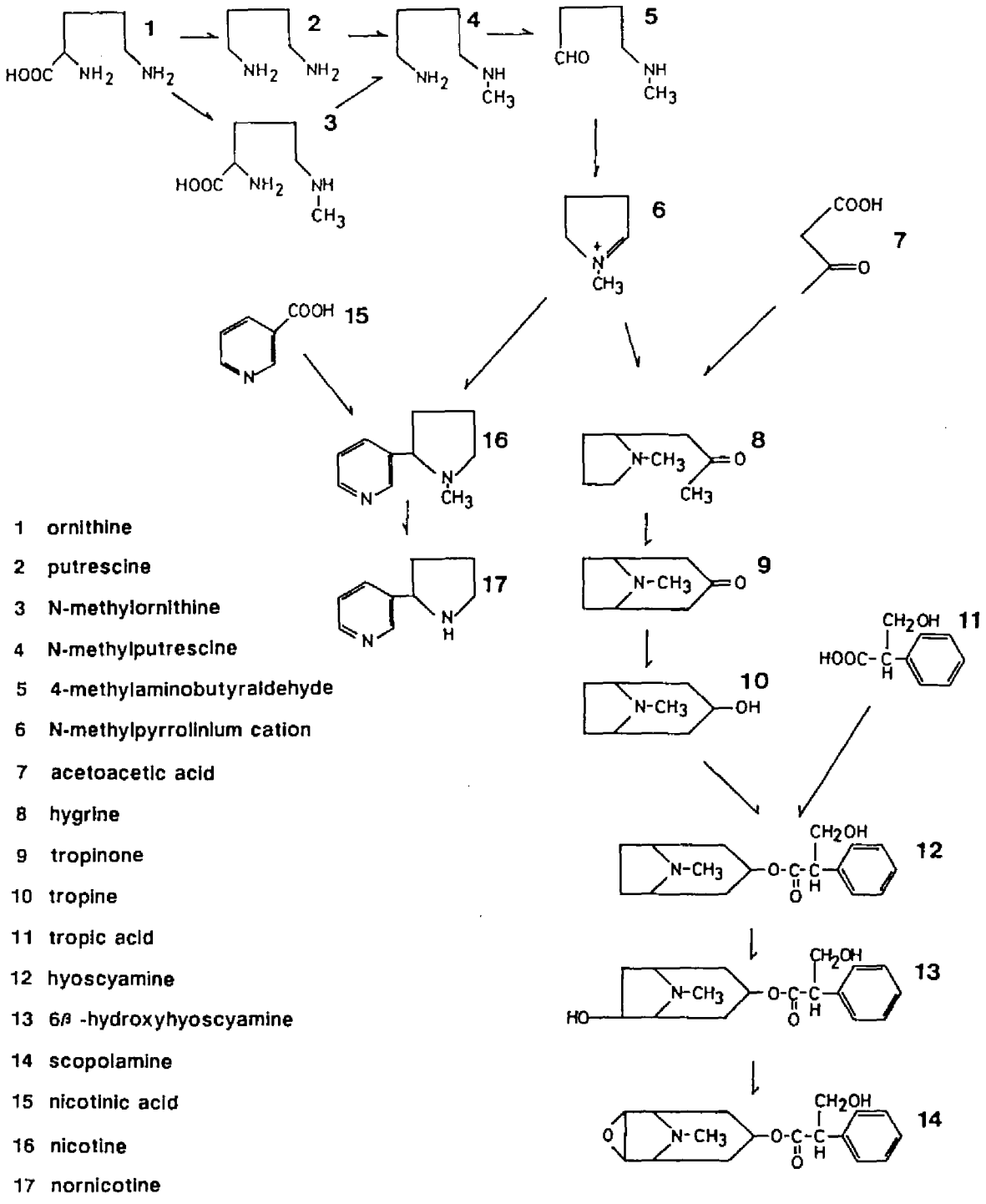


Fig. 1 Biosynthetic pathway of tropane and nicotine alkaloids

CHAPTER I

ALKALOID PRODUCTION IN CULTURED *DUBOISIA* TISSUES

INTRODUCTION

Since the dawn of human history secondary metabolites produced by plants such as alkaloids, terpenoids and pigments have been utilized by man. These compounds are obtained only from plants grown in the field, and the yield in the plants varies depending on geographical source and time of collection. Consequently, some low yield compounds are among the most expensive drugs on the pharmaceutical market (Misawa and Endo 1988). With the progress of plant cell culture techniques, great interest has arisen in the production of these useful compounds by cultured plant cells (Kurz and Constabel 1979, Curtin 1983, Yamada 1984). Development of plant cells with high potential in metabolite production is useful not only for the industrial production of commercially important compounds, but also for studying the biosynthetic pathways of secondary metabolites, and eventually for investigating the regulatory mechanisms of the biosynthesis of these compounds. Recent intensive studies have made it possible to establish some cell lines which produce considerable amounts of secondary metabolites: *Catharanthus roseus* cells producing ajmalicine and serpentine (Zenk et al. 1977), *Coptis japonica* cells producing berberine and its derivatives (Sato et al. 1982, Sato and Yamada 1983), *Euphorbia millii* cells producing antocyanins (Yamamoto et al. 1982) and *Lithospermum erythrorhizon* cells producing shikonine derivatives (Fujita et al. 1985). Enzymatic reactions in alkaloid biosynthesis have been studied with high-producing cell culture systems. Zenk and co-

workers (1985) and Yamada and Okada (1985) have investigated enzymatic reactions involved in isoquinoline alkaloid biosynthesis. The biosynthetic pathway leading to the indole alkaloid ajmalicine was clarified with Catharanthus roseus cell cultures which produced high levels of alkaloids (Scott 1981). Recently the enzymes involved in the biosynthesis of the antineoplastic indole alkaloid vinblastine have also been determined in C. roseus cells (Endo et al. 1987, 1988).

In our laboratory, Yamada and Hashimoto (1982) undertook the investigation of tropane alkaloid biosynthesis with cultured cells of some Solanaceae species. The aim of this study is to explore the potentiality of alkaloid production in cultured tissues of Duboisia species, whose leaves accumulate considerable levels of the tropane alkaloids hyoscyamine and scopolamine.

SECTION 1. Alkaloid Production and Plant Regeneration from *Duboisia leichhardtii* Calluses.

Although there have been some studies on the tissue cultures of *Duboisia myoporoides* and *D. myoporoides* X *D. leichhardtii* sexual hybrids (Sippy and Friedlich 1975, Kitamura et al. 1980, Griffin 1979), little has been reported on cultured cells of *D. leichhardtii*. To my knowledge, a report by Kagei (1980) was the sole study on tissue cultures of this species. Sippy and Friedlich (1975) reported that the calluses of *D. myoporoides* produced hyoscyamine and scopolamine, but other researchers have failed to detect these tropane alkaloids in cultured cells of *Duboisia* species.

In this study I determined the condition for plant regeneration from calluses of *D. leichhardtii* and measured the alkaloid content of the calluses and of the re-differentiated tissues.

MATERIALS AND METHODS

Callus induction A *Duboisia leichhardtii* plant cultivated in a field in Gifu, Japan was used as a source of the explants for callus initiation. Sterilized segments of the leaves , stems, and flower buds were incubated at 25°C on media containing $5x10^{-5}$ M NAA, $5x10^{-6}$ M BA, and 3% sucrose in the light (3000 to 5000 lux) or in the dark. The basal media used were Linsmaier-Skoog (Linsmaier and Skoog 1965), Gamborg B5 (Gamborg 1968), and White (White 1963).

Shoot and root cultures Media used for shoot and root cultures are shown in RESULTS section. Shoot cultures were

maintained under light (3000 to 5000 lux). Adventitious roots were cultured in liquid medium on a reciprocal shaker at 60 strokes/min in the dark.

Cultivation of regenerated plants About 1 month after root initiation, young plantlets were transferred to sterilized soil and cultured under artificial light at 25°C until the roots developed well in the soil. The regenerated plants were cultivated in a green house, which was heated at 15-25°C from December to March.

Extraction for gas-liquid chromatography Freeze-dried samples (ca 200 mg) were powdered and immersed in 5 ml of EtOH-28%NH_4OH (19:1, v/v). After being kept over night at room temperature, the macerated material was centrifuged for 5 min at 2000 rpm. Extraction with the basic alcohol was repeated once more, and the combined alcohol extracts were evaporated to dryness. The residue was taken up in 1 ml HCl (0.1N) and this solution was made alkaline (final pH 10). Then 6 ml of $CHCl_3$ was added. After having been shaken well, the $CHCl_3$ phase was evaporated to dryness, and the alkaloid was dissolved in appropriate volume of 1,4-dioxane:BSA (bis-TMS-acetoamide) (19:1) solution for gas chromatographic analysis.

Gas-liquid chromatography and mass spectrometry Alkaloid contents were measured by gas-liquid chromatography. Chromatographic conditions were a 2.5 m x 3.4 mm glass tube packed with 2% silicon OV-1, a column temperature of 150-238°C (temperature program 6°C/min at 238°C isothermal), helium as the carrier gas at a flow rate of 50 ml/min, and FID as a detector. A capillary column OV-101 (25 m x 0.3mm) was also used in later studies. Tricosane or homatropine was added as the internal standard before injec-

tion for some studies. The mass spectra of alkaloids were recorded with a GC-MS chromatograph. The chromatographic and spectrometric conditions were a 1 m x 3.4 mm glass tube packed with 3% silicon OV-1, a column temperature of 193°C, helium as the carrier gas at a flow rate of 15 ml/min, TMS as the detector, and an ionizing energy of 20 eV. For tobacco alkaloids, 1% silicon OV-101 instead of 3% silicon OV-1 was used and the column temperature was 100-180°C (temperature program, 3°C/min) instead of 193°C isothermal.

In this study no distinction was made between l-hyoscyamine and atropine (d,l-hyoscyamine).

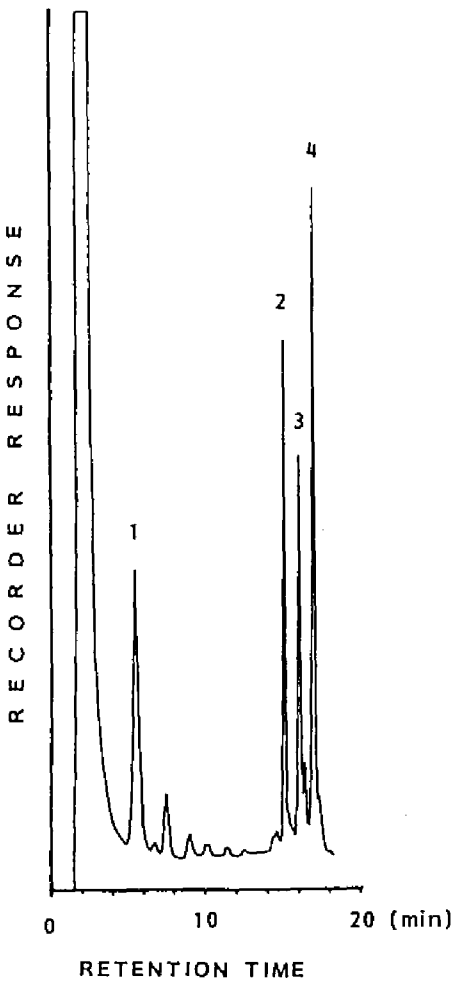
Hyoscyamine biotransformation Hyoscyamine (1 mM, 0.1 mM) was added to the culture media of unorganized callus, shoot-organizing callus, and cultured roots. About 1 g of tissue was incubated in 25ml of medium with hyoscyamine for 4 weeks.

RESULTS

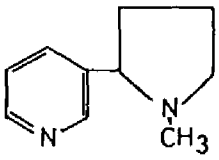
Forty calluses were obtained from 300 explants after 1 month of incubation. Calluses were induced most easily from leaf segments under light on Gamborg B5 media. All calluses formed were transferred to B5 medium containing 10^{-5} M NAA and 10^{-6} M BA, and were maintained on the same medium with transfers at 3 or 4-week intervals. Thirty lines of calluses initiated under light were placed on regeneration medium (B5 medium with 10^{-5} M BA). Upon subcultures, only the green portions of calluses were transferred to fresh medium at 1 month intervals. In 4 months, shoot-organizing calluses were obtained from 2 callus lines. These shoot-organizing callus were subcultured on the regeneration medium with 3 or 4-week intervals. For root formation, the

shoot-organizing tissues were transferred to B5 medium with 10^{-5} M IBA. In 2 to 3 weeks, adventitious roots formed in most of these cultures.

Three months after callus induction, alkaloids in the calluses were analyzed with gas-liquid chromatography. Callus extracts contained small amounts of hyoscyamine (0.002% dry weight) and scopolamine (0.0005% dry weight), bur after

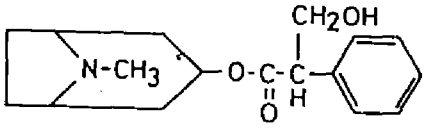


1 Nicotine



2 Homatropine (Internal Standard)

3 Hyoscyamine



4 Scopolamine

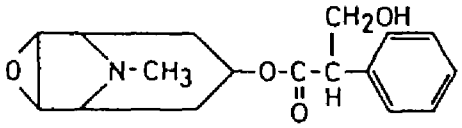


Fig. 1 Gas-liquid chromatogram of an extract of cultured D. leichhardtii roots

one more month of subculture, I could no longer detect hyoscyamine and scopolamine in the cultured cells. The shoot-organizing cultures maintained on the regeneration media also did not contain detectable amounts of hyoscyamine and scopolamine. When adventitious roots were induced in the shoot organizing calluses, hyoscyamine, scopolamine, and nicotine appeared. These tissues grew into whole plants on B5 media without hormones. The regenerated plants (about 1 year old) contained as much alkaloids as the parent plant (the content of hyoscyamine and scopolamine in the leaves of the parent plant were 1.38% and 0.15%, respectively).

This regeneration study showed that root formation in cultured D. leichhardtii cells is intrinsically associated with the alkaloid production. I therefore cultured regenerated adventitious roots, which grew well in the liquid medium containing 10^{-5} M IBA. The content of alkaloids was comparable to that in the roots of the regenerated plants. A typical gas-liquid chromatogram of alkaloids extracted from cultured roots of D. leichhardtii is shown in Fig. 1. The alkaloids in the cultured tissues and the regenerated plants are listed in Table 1.

To examine the ability to transform hyoscyamine to scopolamine, hyoscyamine was fed to unorganized callus, shoot-organizing callus, and cultured roots. After 4 weeks of incubation, alkaloids were analyzed. Unorganized callus had very poor conversion ability, whereas both regenerated tissues converted hyoscyamine added to scopolamine (Table 2). The conversion rates found in shoot-organizing callus and cultured roots when 1 mM hyoscyamine was fed were 2% and 17%, respectively. Tropic acid, the immediate precursor of hyoscyamine, was also fed to unorganized callus, shoot-organizing callus and cultured roots. Tropic acid at 1 mM completely inhibited the growth of all three cultures. Furthermore, neither 1 mM nor 0.1 mM tropic acid showed en-

Table 1. Alkaloid Content of Cultured *D. leichhardtii* Cells and Regenerated Plants

	hyoscyamine (% dry wt)	scopolamine (% dry wt)	nicotine (% dry wt)	scopolamine / hyoscyamine
callus	N.D.[3]	N.D.	N.D.	-
callus with shoots (shoot-organizing callus)	N.D.	N.D.	N.D.	-
callus with shoots and roots[1]	0.36	0.30	0.24	0.8
regenerated plants[2]				
leaves	1.16	0.74	0.02	0.6
stems	0.28	0.15	N.D.	0.5
roots	0.56	0.22	0.33	0.4
cultured roots	0.16	0.37	0.28	2.3

1) analyzed 2-3 weeks after root initiation.
2) ca. 1 year after root initiation.
3) N.D.= not detected.

Table 2. Hyoscyamine Biotransformation

	hyoscyamine added	hyoscyamine (mg/flask) cells	medium	total	scopolamine (mg/flask) cells	medium	total
callus	0 mM	0.00	0.00	0.00	0.00	0.00	0.00
	0.1 mM	0.45	0.02	0.47	0.00	0.00	0.00
	1 mM[1]	1.65	0.23	1.88	0.01	0.00	0.01
callus with shoots	0 mM	0.00	0.00	0.00	0.00	0.00	0.00
	0.1 mM	0.21	0.21	0.42	0.03	0.00	0.03
	1 mM	2.58	2.20	4.78	0.16	0.01	0.17
cultured roots	0 mM	0.21	0.17	0.38	0.66	0.38	1.04
	0.1 mM	0.30	0.11	0.41	0.55	0.22	0.77
	1 mM	1.59	0.67	2.26	1.75	0.55	2.30

1) concentration of 1 mM corresponds to 7.24 mg of hyoscyamine per flask.

hanced tropane alkaloid production (data not shown).

DISCUSSION

Plant tissues known to produce secondary metabolites sometimes lose the ability to produce the metabolites during the process of dedifferentiation, and subsequent undifferentiated growth upon callus induction. This lost ability, generally, can be restored when shoots or roots are redifferentiated from calluses or cell suspension cultures. For example, cardenolide synthesis in Digitalis purpurea (Hirotani and Furuya 1977, Hagimori et al. 1982) and dimeric indole alkaloid synthesis in Catharanthus roseus (Endo et al. 1987) were related to shoot differentiation. On the other hand, quantitative and qualitative expression of ability to form tropane alkaloids has been reported to depend on organogenesis of plants in Datura innoxia (Hiraoka and Tabata 1974), Atropa belladonna (Eapen et al. 1978), and Hyoscyamus niger (Hashimoto and Yamada 1983). Consequently, tropane alkaloid production by callus or cell suspension has not been successful (see Kurz and constabel 1985). This general phenomenon is also true in Duboisia leichhardtii . Unorganized calluses of D. leichhardtii retain the genetic potential to produce tropane and pyridine type alkaloids, but expression is repressed at the dedifferentiated state. When shoots are induced from unorganized callus the ability to convert hyoscyamine to scopolamine appears, and upon root initiation the whole pathway of alkaloid biosynthesis is expressed.

Tropine and nicotine alkaloids are synthesized mainly in the roots of some Solanaceae plants. The alkaloid produced in roots of Datura and Atropa is hyoscyamine, which is converted to scopolamine in the aerial parts of the

plants (Walker and Nowacki 1978). These previous studies to determine the site of alkaloid synthesis in plants were conducted *in vivo* by grafting the aerial parts of alkaloid-producing plants onto alkaloid-free plants, or in the reverse way. By using shoot-organizing callus that corresponds to the aerial parts of a plant and cultured root that corresponds to the roots of an intact plant, I could examine the exact biosynthetic ability of the plant parts separately, thus avoiding the influence of interaction between the two parts of the plant. I found that the site of alkaloid formation in *D. leichhardtii* is similar to the site in *Datura* and *Atropa* i. e. in roots, But in *D. leichhardtii* hyoscyamine can be biotransformed to scopolamine by the root alone. Furthermore, the conversion rate in cultured roots is higher than that in shoot-organizing callus. This result indicates that hyoscyamine is converted to scopolamine in both roots and aerial parts of *D. leichhardtii*, but the main site of the conversion seems to be the roots.

Although *D. leichhardtii* had been known to produce large amounts of tropane alkaloids, Kennedy (1971) demonstrated nicotine alkaloids in roots of this species. I also detected relatively large amounts of nicotine in cultured roots and in intact plant roots.

Cougoul et al . (1979) reported in their study on *D. myoporoides* that younger leaves showed a higher ratio of scopolamine to hyoscyamine than older leaves. The higher ratio of scopolamine to hyoscyamine found in cultured roots than in roots in intact plants may also result from difference in the developmental stages of the roots.

SECTION 2. Alkaloid Production in Cultured Roots of Three Species of *Duboisia* .

Methodology for root culture has long been established even before callus and cell suspension cultures were widely utilized (see review by Butcher and Street 1964). But production of secondary metabolites by root culture has little been studied. The main site of tropane and nicotine alkaloid production in Solanaceae plants is the root. Therefore root culture system in these plants is suitable material for the investigation of alkaloid biosynthesis. In this section the alkaloid spectra of cultured roots derived from calluses of *Duboisia leichhardtii*, *D. myoporoides* and *D. hopwoodii* are described. The alkaloid spectra are compared with those found in intact plants, and culture conditions and selection methods favorable for tropane alkaloid production are discussed.

MATERIALS AND METHODS

Plant materials *Duboisia leichhardtii* plants used in this study were regenerated from 2 to 3 month old calluses as described in section 1. The seeds of *D. myoporoides* were collected from plants cultivated in Barulega, Indonesia. The seeds of *D. hopwoodii* were collected from wild plants in Western Australia and stored in King's Park and Botanic Garden, Australia (*Duboisia hopwoodii* No. 5092). The seeds of *D. myoporoides* and *D. hopwoodii* germinated in October 1981 and December 1981, respectively. The plants were cultivated in a green house , which was heated at 15-25°C from December to March. The plant leaves and roots were harvested in November 1982 for an alkaloid assay.

Callus induction and subculture Sterilized segments of leaves were incubated on Gamborg B5 medium containing $5x10^{-5}$ M NAA, $5x10^{-6}$ M BA, 3% sucrose, and 0.9% agar under light. The callus formed were maintained under the same conditions with 3 week transfers, but the hormone supplement was adjusted to 10^{-5} M NAA and 10^{-6} M BA.

Alkaloid assay and mass spectrometry The alkaloid extraction and quantitative analysis by gas-liquid chromatography were shown in section 1. The mass spectra of hyoscyamine, scopolamine, nicotine and nornicotine were recorded by GC-MS. The conditions for chromatography and spectrometry were also described in section 1. TMS-hyoscyamine: m/e (rel. int.) 361 [M^+] (21), 124 (100), 83 (26), 94 (54), 140 (6). TMS-scopolamine: 375 [M^+] (20), 138 (100), 94 (54), 154 (46), 108 (43). Nicotine: 162 [M^+] (24), 84 (100), 133 (24), 161 (17). Nornicotine: 148 [M^+] (20), 118 (100), 146 (42), 70 (37).

RESULTS AND DISCUSSION

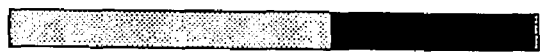
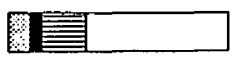
Young calluses of Duboisia species tend to form adventitious roots on the subculture media. This root-differentiating ability, however, decreased during long-term cultures. When the roots formed were excised and cultured in darkness on a reciprocal shaker (60 strokes/min) in liquid medium with 10^{-5} M IBA, the roots showed good growth. The transfers were made every 4 weeks by inoculating the rapidly growing roots (ca 1 cm in length) to a fresh medium.

Normal tropane alkaloids have not been demonstrated in callus tissues of D. myoporoides (Kitamura et al 1980), D. leichhardtii X D. myoporoides hybrids (Griffin 1979), or D. leichhardtii (Kagei 1980). We also found that in calluses

of D. hopwoodii as well as those of the two other Duboisia species, did not produce tropane and pyridine alkaloids. Shoots were regenerated when the calluses were transferred to an agar-solidified medium containing 10^{-5} M BA. In these shoot-organizing cultures of the three Duboisia species, neither tropane nor pyridine alkaloid was detected, but cultured roots differentiated from non-producing calluses produced both type of alkaloids with considerable yields.

The alkaloid spectra of cultured roots subcultured 2-4 times after root initiation were compared with those of leaves and roots of intact plants (Table 1). In the leaves of D. leichhardtii and D. myoporoides, the main alkaloids were the tropane alkaloids hyoscyamine and scopolamine, and nicotine content was very low. The roots of these two species, however, contained relatively large amounts of nicotine. Considering the result that shoot-organizing calluses of Duboisia did not produce detectable amounts of the alkaloids, these data suggest that in D. leichhardtii and D. myoporoides, tropane and pyridine alkaloids are synthesized in the roots exclusively and that selective transportation of tropane alkaloids from the roots to the aerial parts of the plants occurs whereas most of the nicotine remains in the roots. Another possibility is that rapid degradation of nicotine takes place in the aerial parts of these plants. In contrast, the ratio of nicotine alkaloids to tropane alkaloids in the aerial parts of Duboisia hopwoodii is similar to that in the roots, which indicates that the selective transportation of tropane alkaloids suggested for D. leichhardtii and D. myoporoides does not take place in D. hopwoodii. Alternatively, the nicotine is not degraded in the aerial parts of this species. Nornicotine was found only in the leaves of D. hopwoodii. The demethylation reaction from nicotine to nornicotine seems to occur in the

Table 1 Alkaloid spectra of Duboisia

	alkaloid spectra	pyridine alkaloid / tropane alkaloid	scopolamine / hyoscyamine
D. leichhardtii			
plant leaves		0.01	0.64
plant roots		0.42	0.39
cultured roots		1.00	1.24
D. myoporoides			
plant leaves		0.02	2.59
plant roots		1.65	0.63
cultured roots		0.61	3.60
D. hopwoodii			
plant leaves		5.58	0.50
plant roots		3.23	0.56
cultured roots		5.59	0.42

hyoscyamine scopolamine nicotine nornicotine

aerial parts of this species.

The alkaloids produced in cultured roots were similar to those in the roots of intact plants; the contents, however, were lower. That the ratios of scopolamine to hyoscyamine in the cultured roots of D. leichhardtii and D. myoporoides were higher than those in the roots of intact plants might result from the difference in the stage of development between the cultured roots and the intact plant roots. Cultured roots of Hyoscyamus niger derived from suspension cultured cells exhibited higher scopolamine production than normal roots (Hashimoto and Yamada 1983).

Typical time courses for the cultured roots are shown in Fig 1. The doubling times measured for the cultured roots of D. leichhardtii, D. myoporoides and D. hopwoodii were 30, 117 and 73 hr, respectively. Alkaloid production decreased in the early stage of incubation but began to increase when roots reached the logarithmic phase of growth, and it continued after the growth had stopped in D. leichhardtii and D. hopwoodii roots. Especially the amount of scopolamine increased steadily in D. leichhardtii roots, even after day 16, when the amount of hyoscyamine had reached a plateau. This result suggests that the enzyme activity involved in tropane alkaloid biosynthesis was active until the end of the culture period. In contrast, D. myoporoides roots showed relatively stable alkaloid content throughout the culture period. D. leichhardtii and D. myoporoides root released scopolamine to the media, and small amount of hyoscyamine was also discharged from the roots of D. leichhardtii. The alkaloids found in the media, however, comprised only a small proportion of the total alkaloid production in the roots.

Cultured roots of D. leichhardtii showed the highest level of tropane alkaloid production and the most rapid growth among the three Duboisia species. Therefore I under

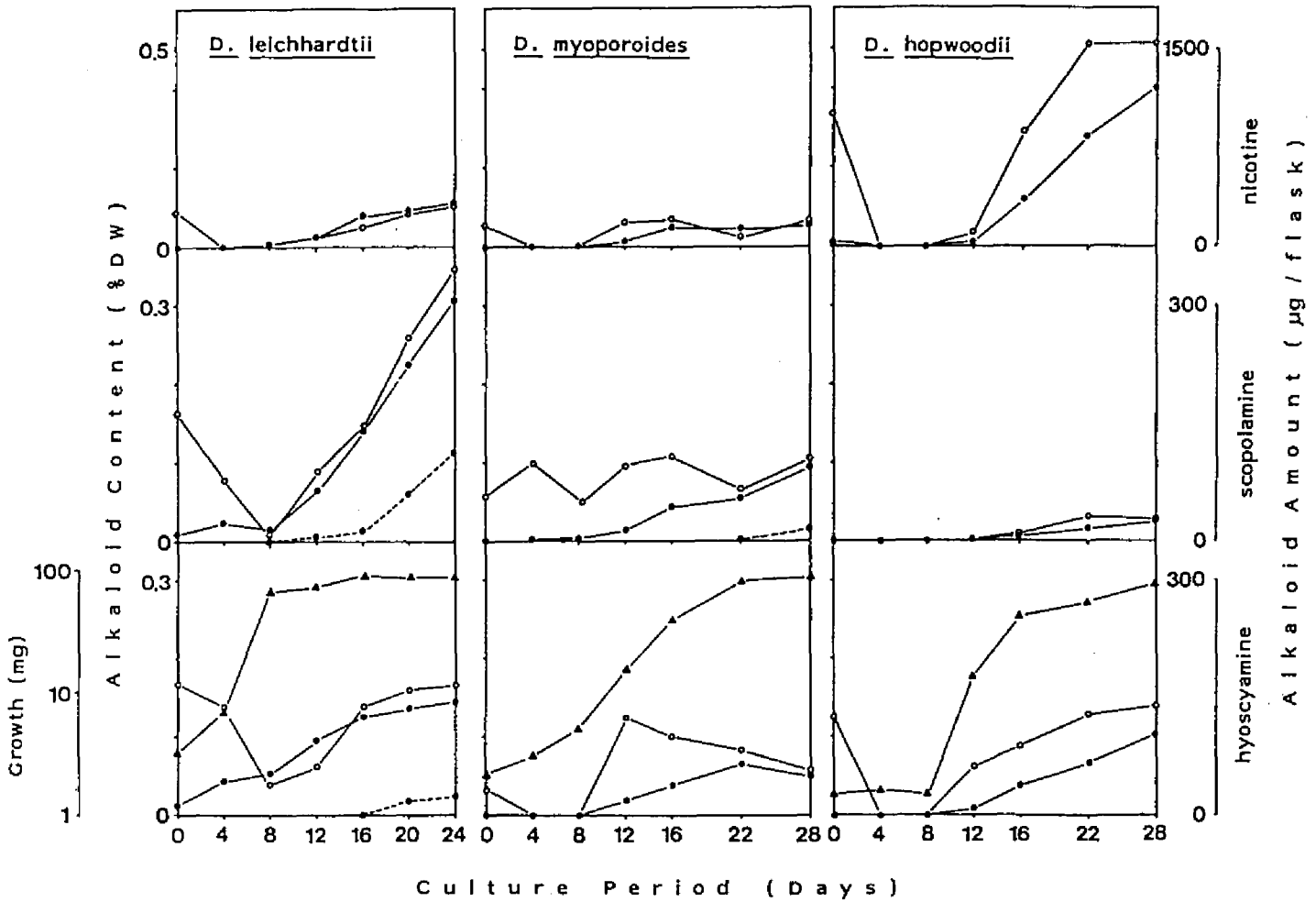


Fig. 1 Time Courses of Growth and Alkaloid Production in Cultured Duboisia Roots

Symbols denote the alkaloid content (—•—, % DW), amount in roots (—•—, ug/ flask), amount in medium (--•--, ug/ flask) and growth (—▲—, mg DW).

took the preliminary selection of D. leichhardtii roots for high tropane alkaloid production. First, all the root lines obtained were analyzed, then the root lines with the highest contents were selected, and 10 roots (ca 1 cm) of these lines were incubated in four or five flasks. The roots were cultured for 4 weeks, after which they were analyzed and re-selected. We obtained a high alkaloid-producing line by repeating this selection procedure ten times. The contents of hyoscyamine and scopolamine in this line were 0.53 and 1.16% on a dry weight basis, respectively (Table 2). These values were ca three times more than the contents of low-producing lines. The sum of hyoscyamine and scopolamine contents in this line is twice as high as that in roots of intact plants and is almost comparable to that in leaves of intact plants. Only a few root cultures, so far reported, have shown tropane or nicotine alkaloid production comparable to the levels of whole plants (Solt 1957, Solt et al. 1960, Mitra 1972, Hashimoto et al. 1986).

I also studied which culture conditions of D. leichhardtii roots were favorable to alkaloid production. A high sucrose concentration in the medium stimulated nicotine production; the nicotine content in roots cultured in B5

Table 2.

Selection of Cultured Roots for High Alkaloid Production

		hyoscyamine (% DW)	scopolamine (% DW)
before selection	content max.	0.22	0.45
	min.	0.04	0.06
after selection	content max.	0.53	1.16
	min.	0.14	0.33

Table 3. Effects of aeration on alkaloid-production in cultured *D. leichhardtii* roots

Culture vessel	Growth*	Roots above water surface	Hyoscyamine		Scopolamine		Nicotine	
			μg/g fr. wt cell	μg/vessel	μg/g fr. wt cell	μg/vessel	μg/g fr. wt cell	μg/vessel
300 ml flask	1.84	+ +	214	394	255	470	543	999
100 ml flask	3.90	+ +	201	620	289	893	304	939
50 ml flask	4.91	+	30.8	151	85.3	419	52.3	257
Test tube	0.69	—	31.9	22	53.6	37	—	n.d.

*g/culture vessel.
Average inoculum was 0.20 g/culture vessel. Medium volume was 25 ml.

medium supplemented with 5% sucrose was three times higher than that in the 3% sucrose medium. In Linsmaier-Skoog medium, D. leichhardtii roots produced 1.8 times more nicotine than in B5 medium. These change, however, did not affect the production of hyoscyamine and scopolamine, which are commercially more important than nicotine. Changes of neither the mineral salt nor vitamin concentration in B5 medium (x3, x1, and x1/3 concentration of standard medium) showed significant effects on the alkaloid production (data not shown).

The effects of aeration on alkaloid production in D. leichhardtii roots are shown in Table 3. Roots were inoculated into 25 ml liquid B5 medium with IBA in Erlenmeyer flasks of different volumes (300, 100, and 50 ml) and in the test tubes of 28 mm diameter. A larger flask has a larger water surface than a small flask when the same volume of medium is in it; therefore the former is better aerated. In the test tubes, the roots sank to the bottom and both the growth rate and the alkaloid production were low. The roots in the 50 ml flasks showed the best growth but the 100 ml flasks were the most favorable for alkaloid production. Thus the optimum aeration rate for alkaloid production may be higher than that for growth. Tropane alkaloid production in the 300 ml flasks was considerably lower than that in 100

ml flasks. In the 300 ml flasks, the growth may have been suppressed by mechanical injury caused by strong shaking.

As a potential material for production of tropane alkaloids, I believe that the cultured root of Duboisia leichhardtii is the most promising because of its rapid growth and high alkaloid content.

SECTION 3. Non-Enzymatic Synthesis of Hygrine from Acetoacetic Acid and from Acetonedicarboxylic Acid

Hygrine, an intermediate in biosynthesis of tropane alkaloids, has been suggested to be synthesized *in vivo* by the coupling of MABA with an unknown counterpart, possibly, acetoacetic acid (Leete 1980), acetoacetyl CoA (O'Donovan and Keogh 1969) or acetonedicarboxylic acid (Robinson 1917). MABA, which is derived from the amino acids ornithine or arginine, exists in equilibrium with N-methylpyrrolinium cation. Acetoacetic acid and acetonedicarboxylic acid respectively were reported to couple with MABA under physiological conditions (Leete 1980, Anet et al. 1949), although the parameters and mode of action in this reaction have not been sufficiently explored. The hygrine formed is incorporated into the tropine moiety of hyoscyamine (O'Donovan and Keogh 1969, McGaw and Wooley 1979a).

In *Nicotiana* species when MABA is coupled with nicotinic acid, nicotine is synthesized. As *Duboisia* species produce both tropane and nicotine type alkaloids, regulatory mechanism in biosynthesis of tropane and nicotine alkaloids at this branching point is particularly interesting. I have studied biosynthesis of hygrine, the first intermediate leading to tropane alkaloids. In this section, I present the non-enzymatic formation of hygrine from either acetoacetic acid or acetonedicarboxylic acid in detail and discuss a possible *in vivo* process involved in hygrine synthesis.

MATERIALS AND METHODS

Chemicals MABA was obtained by acid hydrolysis of

γ-methylaminobutyraldehyde diethylacetal synthesized from Formyl-γ-aminobutyraldehyde diethylacetal (Sigma) after Mizusaki et al. (1968). MABA formed was purified through Dowex 50 wx8 as described by Feth et al. (1985). Acetyl CoA, Acetoacetyl CoA and acetoacetic acid were purchased from Sigma.

Cell-free preparation Freeze dried roots of Duboisia leichhardtii and Hyoscyamine albus (Hashimoto et al. 1986) cultured in vitro were ground in a chilled mortar with 2 volumes of potassium phosphate buffer (pH 7.5, 100 mM), 3 mM dithiothreitol, and the same volume of polyvinylpolypyrollidone. After the centrifugation at 10,000xg for 20 min, the protein was precipitated with 80% saturated $(NH_4)_2SO_4$, then collected with another centrifugation (10,000xg, 20min). The pellet was dissolved in Tris/HCl buffer (pH 7.0, 50 mM) and desalted with Pd-10 column (Pharmacia). About 1 mg protein was applied to a reaction mixture.

Reaction condition In Tris/HCl buffer (pH 7.0, 50 mM), 0.1 mg acetoacetic acid and 0.5 mg MABA was incubated at 30°C for 4 hr in a standard experiment.

Detection and identification of hygrine The reaction mixture was made alkaline with 50 ul 28%NH_4OH, and hygrine was extracted with 5 ml CH_2Cl_2:propanol (85:15) (Hartmann et al. 1986). The solution was condensed to 1.0 ml at 30 C in reduced pressure then applied to gas chromatography (see previous sections).

Root culture of tobacco Aseptically germinated seedlings of Nicotiana tabacum c.v. Samsun were inoculated in liquid media containing minerals and vitamins of B5 medium, 3% sucrose and 10^{-5} M indolebutyric acid. The resulting

root cultures have been maintained on a rotary shaker in the dark with monthly subculturings.

Feeding experiments Acetoacetic acid or acetonedicarboxylic acid (10 mM) was added to 2 week old roots (ca 1 g fresh weight in 10 ml medium). The roots and media were analyzed 24 hr after feeding. The root harvested were freeze dried and the alkaloids were extracted with EtOH:NH_4OH (19:1). The solution was evaporated to dryness and the residue was taken up in 0.1 N HCl. Alkaloid extraction from this solution and the culture media used were the same as described above.

RESULTS

Chemical coupling of acetoacetic acid and MABA Acetoacetic acid and MABA were incubated under a condition described in MATERIALS AND METHODS section. After 24 hr, 58% of the acetoacetic acid was incorporated to hygrine (Fig. 1). In order to determine how much substrates were left in the reaction mixture after 24 hour incubation, acetoacetic acid and MABA were separately added to the mixtures. A further addition of acetoacetic acid to the reaction mixture increased hygrine yield, but an addition of MABA did not, suggesting that no acetoacetic acid was left in the reaction mixture after 24 hr of incubation. Cell-free preparations from root cultures, which produce high level of hyoscyamine and scopolamine, did not promote hygrine formation (Fig. 1).

pH dependence of hygrine formation The maximum amount of hygrine was formed at pH 9 (Fig. 2). The decrease in hygrine yield at higher pH can be explained by alkaline

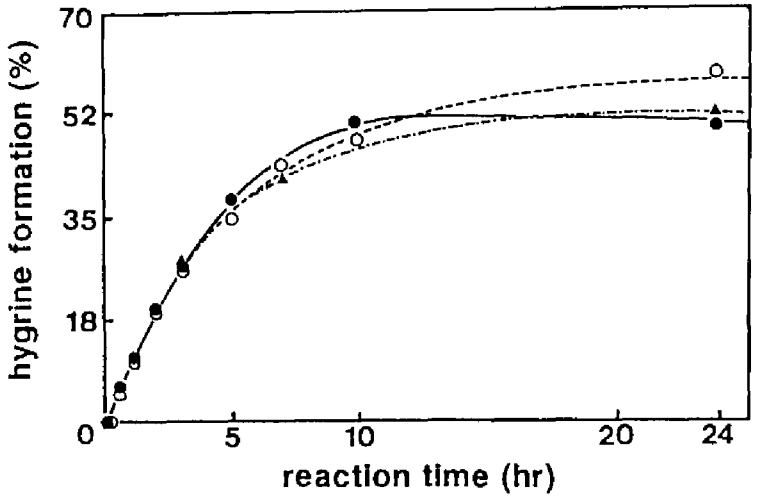


Fig. 1 Time course of hygrine formation from acetoacetic acid and γ-methylaminobutyraldehyde with cell free extract from cultured roots of Duboisia leichhardtii, —●—; Hyoscyamus albus, -·-▲-·-; or without enzyme, ---o---.

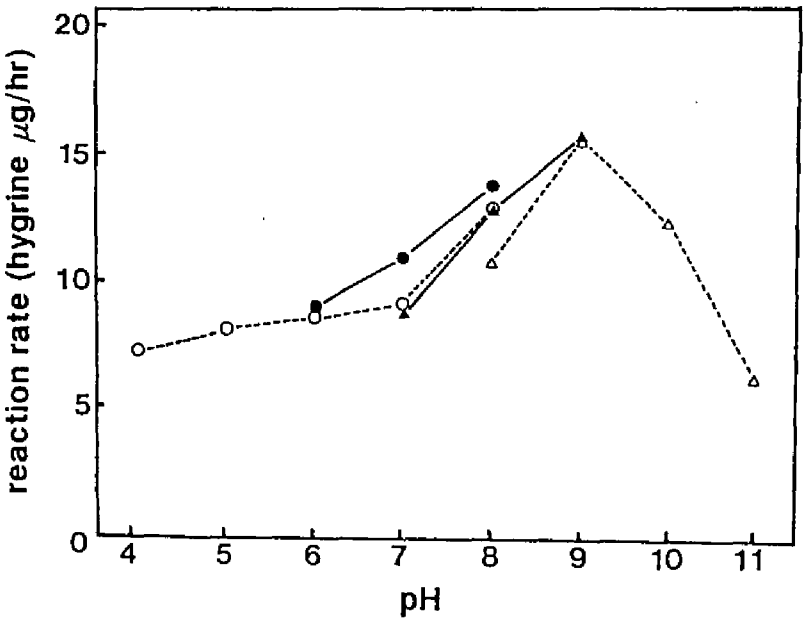


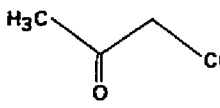
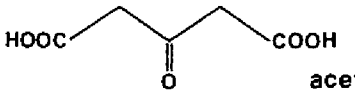
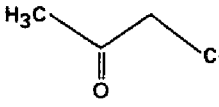
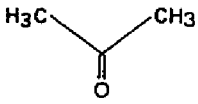
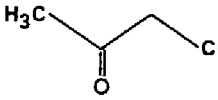
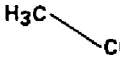
Fig. 2 Effect of pH on hygrine formation. Buffers used were; HEPES 50 mM, —●—; Tris/HCl 50 mM, —▲—; citric acid/Na_2HPO_4 100 mM, ---o---; NH_4OH/NH_4Cl 100 mM, ---△---.

labile nature of MABA. MABA was totally decomposed after a 4 hr incubation without acetoacetic acid at pH 10 and 11.

Hygrine formation from analogues of acetoacetic acid

Several analogues of acetoacetic acid and CoA derivatives were incubated with MABA in the presence or absence of cell-free extract of *D. leichhardtii* root culture (Table 1). Acetonedicarboxylic acid (3-oxoglutaric acid) gave a considerable amount of hygrine, though no cuscohygrine was detected, in contradiction to the report by Anet et al. (1949). A trace amount of hygrine was synthesized from acetone. Acetoacetyl CoA also gave a small amount of

Table 1 **HYGRINE FORMATION FROM ACETOACETIC ACID ANALOGUES**

compound	relative hygrine yield (%)
 acetoacetic acid	100
 acetonedicarboxylic acid	35
 methyl acetoacetate	NOT DETECTED
 acetone	TRACE
 acetoacetyl CoA	8
 acetyl CoA	NOT DETECTED

hygrine, which might be due to the spontaneous breakdown of the compound to acetoacetic acid and CoA-SH. This decomposition could be followed by the decrease in UV absorbance at 303 nm due to thioester-specific enolate anion (Stern 1955). In any case, the crude extract from Duboisia root culture did not enhance hygrine yield.

Table 2. Effect of metal ions on hygrine formation

compound	(mM)	acetoacetic acid (%)[1)]	acetonedicarboxylic acid (%)[1)]
none	-	100	100
NaCl	10	101	113
K_2SO_4	10	142	92
$(NH_4)_2SO_4$	10	111	95
$MgCl_2$	10	172	76
$CaCl_2$	10	133	99
$MnSO_4$	2	153	122
$FeSO_4$	0.5	154	133
$FeCl_3$	0.5	118	272
$CoCl_2$	0.5	129	125
$NiCl_2$	0.5	167	144
$CuSO_4$	0.5	200	234
$Zn(NO_3)_2$	0.5	164	140
$HgCl_2$	0.5	85	123
$Pb(NO_3)_2$	0.5	111	159

1) Values are expressed relative to the yield without any metal ion.

Effects of metal cations on hygrine synthesis The effect of metal cations on hygrine synthesis was examined when these were added to the reaction mixture (Table 2). Some divalent cations stimulated hygrine formation. Copper ion doubled hygrine synthesis from either acetoacetic acid or acetonedicarboxylic acid. Ferric ion markedly enhanced hygrine yield from acetonedicarboxylic acid, but did not change the yield from acetoacetic acid.

Feeding of acetoacetic acid and acetonedicarboxylic acid to cultured root of tobacco Tobacco alkaloids such as nicotine share a common precursor, MABA, with tropane alkaloids.

Acetoacetic acid and acetone-dicarboxylic acid separately were fed to a root culture of tobacco, which produced a considerable amount of nicotine but not tropane

Table 3. Feeding of keto acids to cultured tobacco root

	hygrine formed (μmol/g dry wt)	
	roots	media
Control	N.D.	N.D.
acetoacetic acid	4.3	N.D.
acetonedicarboxylic acid	0.35	N.D.

N.D. = not detected.

Roots were incubated for 24 hr with 10 mM acetoacetic acid or acetonedicarboxylic acid.

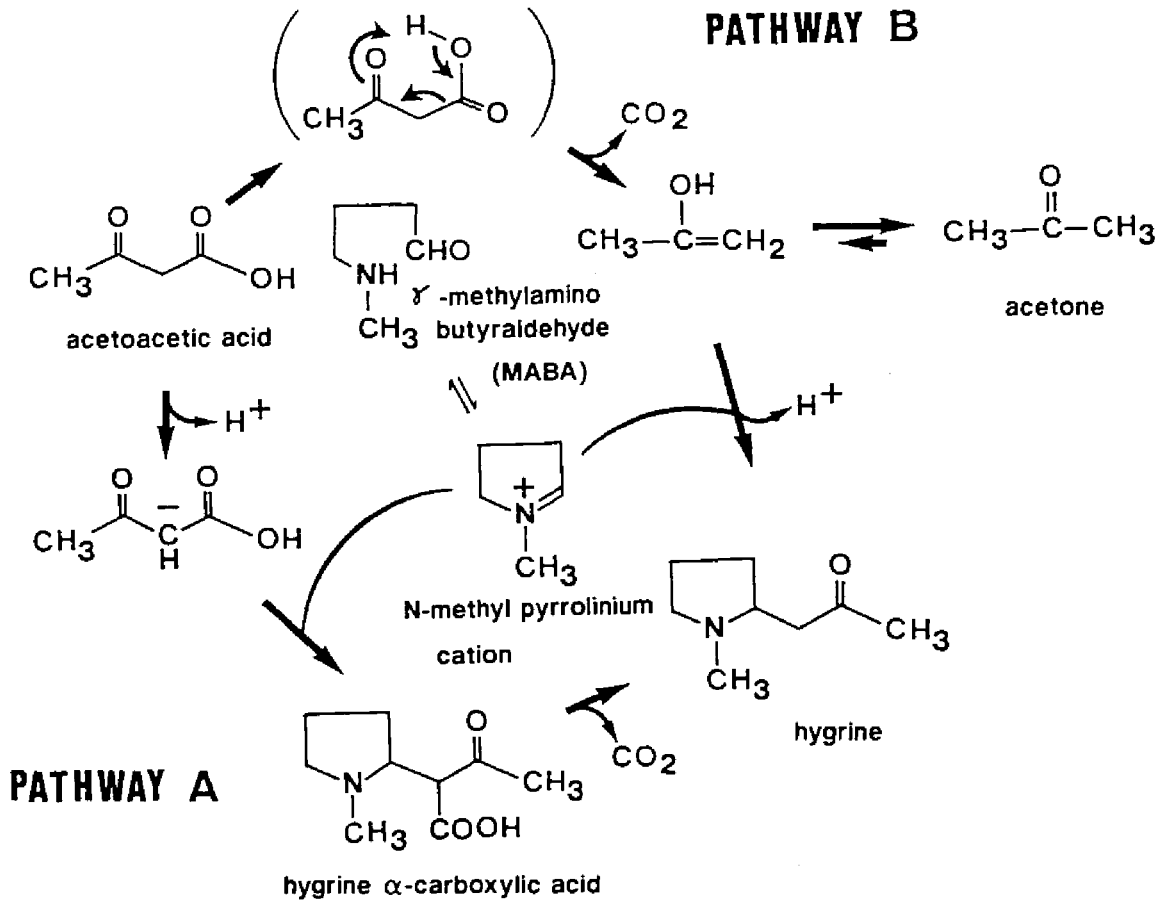
alkaloids, including hygrine. Hygrine was detected when either keto acid had been fed to the tobacco roots (Table 3).

DISCUSSION

In this section, I have shown that non-enzymatic formation of hygrine takes place in physiological pH and temperature. The result of feeding of acetoacetic acid to tobacco roots suggests the possibility of non-enzymatic hygrine formation in vivo.

Two hypothetical processes for the coupling of acetoacetic acid and MABA are proposed. Acetoacetic acid may lose proton to form enolate anion (pathway A in scheme 1) (El-Olemy et al. 1965) or it can decompose to carbon dioxide and an enole (2-hydroxypropene) which is easily turned into acetone (pathway B in scheme 1) before Mannich condensation with MABA takes place.

Formation of a trace amount of hygrine from acetone suggests the possibility of pathway B. The hygrine yield from acetone was only one thousandth the yield from acetoacetic acid (data not shown), indicating that acetone formed from the enole can turn back into the enole with a very low rate. This may explain the result of in vivo feeding (McGaw and Wooley 1979b) in which sodium [4-^{14}C]acetoacetate was incorporated into only C4 position of hygrine. If pathway B is indeed the process in vivo, a putative intermediate, hygrine-α-carboxylic acid is not involved in this reaction, although further experiments are needed to establish the actual in vivo pathway. From acetonedicarboxylic acid, hygrine may be formed with similar process. But in this case second decarboxylation must be considered.



Scheme 1

Although I can not deny the possible involvement of an enzyme system such as acetoacetic acid decarboxylase in hygrine formation, no indication for the involvement of any enzyme could be obtained from this study. Acetoacetic acid and acetonedicarboxylic acid are two possible counterparts to MABA for the formation of hygrine in vivo because they both formed considerable amounts of hygrine when combined with MABA under physiological pH and temperature. Feeding experiments indicated that endogenous MABA produced by

tobacco root cultures could couple with exogenously supplied acetoacetic acid or acetonedicarboxylic acid to form hygrine. Since it is plausible to assume that the tobacco root, which produces no tropane alkaloids does not have an enzyme which catalyzes hygrine formation, the coupling observed must result from a non-enzymatic process. This suggests that, if pools of either acetoacetic acid or acetonedicarboxylic acid and of MABA are present together, non-enzymatic hygrine formation takes place *in vivo*. The presence or absence of an available pool of these keto acids may result in the difference in alkaloids formed in tobacco and in plants which produce tropane alkaloids (*Duboisia*, *Hyoscyamus*, *Datura*, *Atropa*, etc.).

In animals acetoacetic acid is formed from acetoacetyl CoA with CoA transferase, deacylase, or activating enzyme (Stern 1955). Acetoacetyl CoA, which can be formed from two acetyl CoA (thiolase reaction), is involved in terpenoid synthesis and the degradation of fatty acids and some amino acids (leucine, phenylalanine, tyrosine, tryptophane and lysine). Acetonedicarboxylic acid is formed in the course of citric acid degradation by some micro organisms (Walker et al. 1927, Butterworth and Walker 1929).

Although the metabolism of these keto acids has been partly revealed in animal and micro organism systems, in higher plants it is largely unknown. Carbons 2, 3, and 4 of tropane ring has demonstrated to be derived from acetic acid in *Datura metel* roots (Kaczkowski et al. 1961), while labeled citric acid was not incorporated into the tropane ring (Robertson 1960). It is essential to study the metabolism of the ketoacids in plants in order to obtain a clearer understanding of tropane alkaloid biosynthesis because these compounds are key intermediates which link primary and secondary metabolism in plants which produce tropane alkaloids.

CHAPTER II

SOMATIC HYBRIDIZATION OF DUBOISIA AND NICOTIANA

INTRODUCTION

Since the development of an efficient fusion method by the use of polyethylene glycol (Kao and Michayluk 1974, Wallin et al. 1974), the list of somatic hybrids has steadily increased. Although there is an increasing interest in protoplast fusion, studies of metabolite synthesis in somatic hybrids have been rare. To date, most studies on protoplast fusion of higher plants have been conducted for the purpose of plant breeding to improve agronomical traits, and metabolite production by somatic hybrid cells has little been explored, even though hybridoma of mammalian cells have been widely studied and commercialized in the field of animal tissue culture.

Research on protoplast fusion of plant cells which produce large amounts of secondary metabolites may be useful not only for industrial production of commercially important compounds, but also for investigating the biosynthesis of those metabolites. It is especially interesting to study the expression of biosynthesis of metabolites whose synthesis requires intercellar compartmentation or that is synthesized organ specifically, after metabolite-producing cells are fused with other cells.

Besides gene transformation by the Ti plasmid and other vector systems, protoplast fusion is one of the important techniques for introducing foreign genetic materials into plant cells (Carlson 1972, Melchers et al. 1978, Schieder 1978, Zelcer et al. 1978, Gleba and Hoffmann 1979). In contrast to transformation by DNA vectors or by direct gene

transfer, protoplast fusion is suited for introducing multi-gene encoded traits. Successful transfer of disease resistance genes by protoplast fusion has recently been demonstrated in potato (Austin et al. 1985, 1988; Helgeson et al. 1986; Gibson et al. 1988) and in eggplant (Gleddie et al. 1985, 1986). The somatic hybrids of taxonomically remote species generally have asymmetric genome constitution. Elimination of chromosomes from either parental species seems to be a common phenomenon among intergeneric and interfamilial hybrids (Kao, 1977; Krumbiegel and Schieder, 1979; Gleba and Hoffmann, 1978; Gleba et al. 1982), which may result in multi-gene transfer from one species to another. Besides this spontaneous asymmetrization, asymmetric hybridization with gamma-ray irradiated or chemically inactivated protoplasts, originally developed for the transfer of cytoplasmic genome (Zelcer et al. 1978, Aviv and Galun 1980, Menczel et al. 1983, Sidorov et al. 1981), has also been investigated for multi-gene incorporation (Dudits et al. 1980, Gupta et al. 1982, Somers et al. 1986, Imamura et al. 1987). Studies on fusion of metabolite-producing cells may facilitate genetic analysis of the multi-gene transformation which can be achieved by protoplast fusion, because secondary metabolites are generally produced through multi-step biosynthetic pathways which are often species or genus specific. In other words, synthesis of a secondary metabolite in a hybrid cell indicates that the complete set of enzymes on the pathway leading to the metabolite is present in the cell; therefore the production of the metabolite serves as a genetic marker for the group of genes which encodes the series of enzymes involved in the biosynthesis of the metabolite.

In this chapter, I describe somatic hybridization of Duboisia, which produces both tropane and nicotine alkaloids, and Nicotiana, which produces only nicotine

alkaloids. Alkaloid biosynthesis in the somatic hybrids is explored.

Spontaneous asymmetrization of the parental genome as mentioned above and chromosomal instability are frequently observed in intergeneric somatic hybrids of the Solanaceae. The genetic instability in the Duboisia + Nicotiana hybrid cells is also extensively investigated in this chapter.

SECTION 1. An Intergeneric Hybrid Cell Line of *Duboisia hopwoodii* and *Nicotiana tabacum*

In this section, I report the results of somatic hybridization between *Duboisia hopwoodii* and *Nicotiana tabacum* L. var. Samsun. Chromosomes of *Duboisia* are small and therefore easily distinguished from the larger chromosomes of *N. tabacum*.

Visual selection of heterokaryons after protoplast fusion was first reported by Kao (1977) on the basis of the presence of both green chloroplasts from mesophyll protoplasts and dense cytoplasm from suspension protoplasts. Heterokaryons selected have previously been isolated with micropipettes and cloned individually in small amounts of medium (Kao, 1977; Gleba and Hoffmann, 1978) or have been incubated with nurse cells (Menczel et al., 1978; Hein et al., 1983). In this study, I adopted the method of picking up individual hybrid cells by using a micropipette connected to a manipulator, and then culturing the single cells with nurse cells (Yamada and Morikawa, 1985).

Material and Methods

Preparation of Protoplasts A cell suspension of *Duboisia hopwoodii* (line Dh-1S) was initiated from seedling-derived callus and had been maintained in darkness for about 2 years on Gamborg B5 medium (Gamborg et al., 1968) containing 10^{-5} M NAA, 10^{-6} M BA, and 3% sucrose with weekly transfers. Protoplasts of *D. hopwoodii* were obtained from the cell suspension 3 to 5 days after subculture with an enzyme solution containing 4% Cellulase

Onozuka RS, 2% Macerozyme R-10 (both from Kinki Yakult Mfg. Co, Nishinomiya, Japan), 4% Driselase (Kyowa Hakko Co, Tokyo, Japan), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co, Tokyo, Japan), 20 mM MES, 5 mM $MgCl_2$ and 0.6 M sorbitol. Mesophyll protoplasts of Nicotiana tabacum were obtained from young tobacco leaves with an enzyme solution containing 1% Cellulase Onozuka R-10 (Kinki Yakult), 0.5% Macerozyme R-10, 20 mM MES, 5 mM $MgCl_2$, and 0.6 M sorbitol. The enzyme treatment was continued for 3 to 5 hours in the dark at 25°C. Subsequently the protoplast suspensions were filtered through 62 μm nylon mesh, and the filtrate was washed twice with the protoplast culture medium. When the protoplast suspension contained debris even after washing, the suspension was purified using Percoll (Pharmacia) density-gradient centrifugation. Protoplasts from Duboisia suspensions could be collected on the surface between the culture medium containing 10% (v/v) Percoll and the medium containing 20% Percoll with centrifugation (1,000xg, 15min). Samely tobacco mesophyll protoplasts could be packed on the surface between the medium with 30% Percoll and the medium with 50% Percoll.

Protoplast Fusion Fusion treatment was modified after Kao and Michayluk (1974). About 200 μl of a mixed protoplast suspension (with a tobacco, Duboisia ratio of 3:1, and a final population of $3x10^5$ protoplasts/ml) was transferred to a 3.5 cm plastic petri dish, and the protoplasts were allowed to settle for 5 min. Then 6 to 8 drops of 40% PEG (#1540) solution was added to the protoplast mixture, and the protoplasts were incubated in the PEG solution for 1 to 3 min. Next, PEG solution was removed carefully with a small pipette and 0.2 ml of washing solution containing 0.3 M glucose, 50 mM $CaCl_2.2H_2O$ and 50 mM glycine (pH 10.5) was added. After removing the

washing solution, 1 ml of the protoplast culture medium was added (8p medium, Kao and Michayluk, 1975, containing $5x10^{-5}$ M NAA, 10^{-5} M BA, 0.55 M glucose and 5% coconut milk)

Isolation of Heterokaryons and Single Cell Culture

Heterokaryons were identified microscopically on the basis of the presence of both green chloroplasts (from mesophyll protoplasts) and thick cytoplasmic strands (from protoplasts of suspension cultures), and they were isolated by capillary pipette (tip diameter ca. 150 μm) connected to a micromanipulator. Isolated heterokaryons were incubated in a nylon mesh (pore size, 20 μm) cylinder of diameter 4 mm and height 4.5 mm fixed to the bottom of a 3.5 cm plastic petri dish by a drop of 1% agar solution (Fig. 1). Around this cylindrical chamber, 1.5 ml of tobacco mesophyll protoplasts (10^5 protoplasts/ml) were inoculated as nurse cells. Culture medium can permeate into the chamber freely, but tobacco protoplasts remain outside of the chamber.

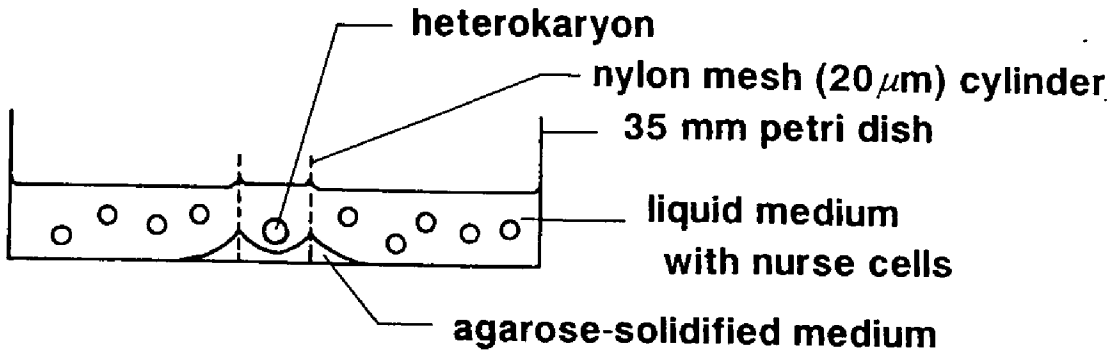


Fig. 1 **nurse culture system for single heterokaryons**

Isozyme Analyses and Cytological Investigations The putative hybrid callus, the parent callus of Duboisia (Dh-1S) and a tobacco callus (T-1104) derived from mesophyll protoplasts were used in this study. For isozyme analyses, cell-free extracts were prepared by grinding the cells in a chilled mortar with cold extraction buffer (50 mM tris/HCl pH 7.5). Electrophoretic conditions and staining methods used were in accordance with Wetter (1982). For cytological studies, callus tissues were pretreated with 0.05% colchicine, fixed in a 1:3 ratio of acetic acid and ethanol, and stained with basic fuchsin.

Analysis of Fluorescent Compounds Freeze dried callus (ca. 200 mg) was kept in 3 ml acetone overnight, and 1 to 4 µl of the extract was spotted on a silica gel plate (60 F254S, Merck). The solvent system for development was n-BuOH:AcOH:H_2O = 10:3:4. Fluorescence was observed under UV light (366 nm).

Results

Isolation of heterokaryons was carried out over a period of 2 to 48 hours after the fusion treatment. A total of 280 fused cells were isolated, among which 80 cells were cultured individually in nylon mesh chamber (i.e. one cell per chamber) and, for the remaining cells, with several cells per chamber. Heterokaryons inoculated in the chambers were cultured in the dark at 25°C. About half of the fused cells isolated were dead within 1 day of culture. On the 10th day of culture in nylon mesh chambers, half of the nurse culture was replaced with a fresh medium containing a reduced concentration of glucose (0.3 M). By this time, some of the surviving cells showed budding and expansion.

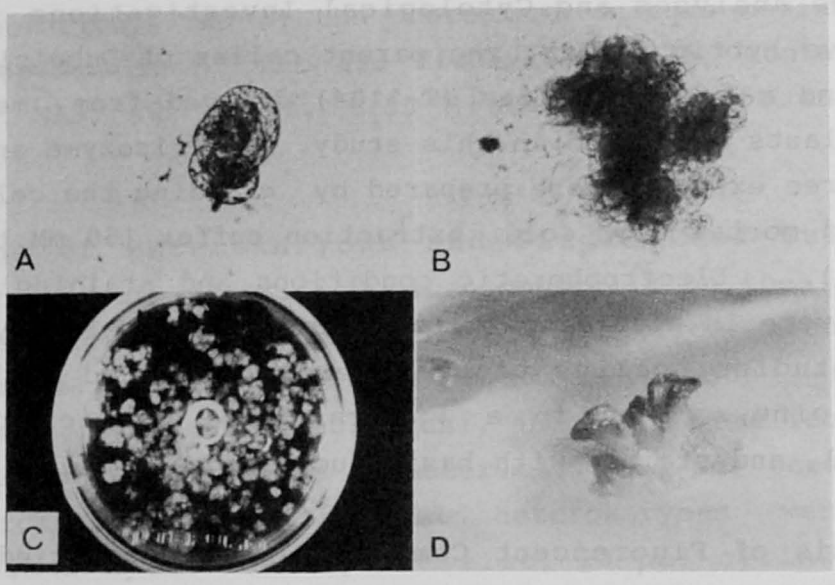


Fig. 2: A hybrid colony (HT-1031) from *Duboisia hopwoodii* + *Nicotiana tabacum* on the 5th day (A), 19th day (B), and 50th day (C) of the culture in a nylon mesh chamber. (D); a regenerated shoot (tobacco type).

Although most failed to develop into colonies, one individually cultured cell divided normally and developed into a colony (Fig 2). On the 20th and 35th day of culture, the same medium was added in the same way.

After 50 days of culture the putative hybrid colony (HT-1031) was removed from the chamber with forceps and transferred to a petri dish with 0.2 ml B5 medium containing $5x10^{-5}$ M NAA, 10^{-5} M BA, and 3% sucrose. Ten days and 20 days after the transfer, 0.3 ml and 0.5 ml of the same medium were added, respectively. After 10 more days of culture, this colony was transferred to B5 medium containing 10^{-5} M NAA, 10^{-6} M BA, 3% sucrose, and 0.9% agar in a 100 ml Erlenmyer flask and maintained in the same medium under light (3000 to 5000 lux). The surface of HT1031 callus tended to turn brown at later stages of culture, and purple spots appeared. The purple spots, which might be due to the production of anthocyanin, were also observed on

Duboisia callus.

Eight to nine months after fusion, electrophoretic separations of peroxidase and acid phosphatase were carried out. The isozyme analyses in HT-1031 showed intermediate patterns between those of parental species (data not shown).

Another indication of the hybrid nature of the callus was provided by the analysis of fluorescent compounds. When callus was put under UV light, tobacco and HT1031 showed blue fluorescence, while Duboisia did not. TLC analysis of the acetone extracts of these calluses showed that under UV light, tobacco and HT1031 had two strong blue spots (B1 and B2 in Fig 3), and that Duboisia showed only a weak B2 spot. In addition, Duboisia callus showed a yellow fluorescent spot (Y in Fig 3), which was found also in HT1031 but not in tobacco callus.

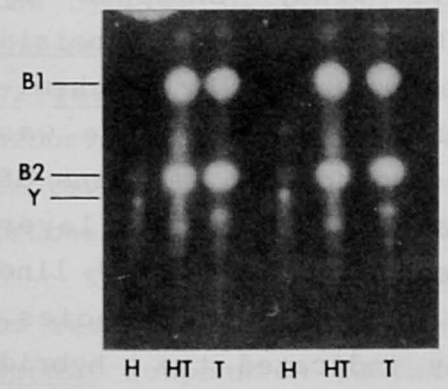


Fig. 3: Thin layer chromatography of fluorescent compounds. *Duboisia hopwoodii* (H), *Nicotiana tabacum* (T), the hybrid HT-1031 (HT). Blue (B1, B2) and yellow (Y) fluorescences were observed under UV light (366 nm).

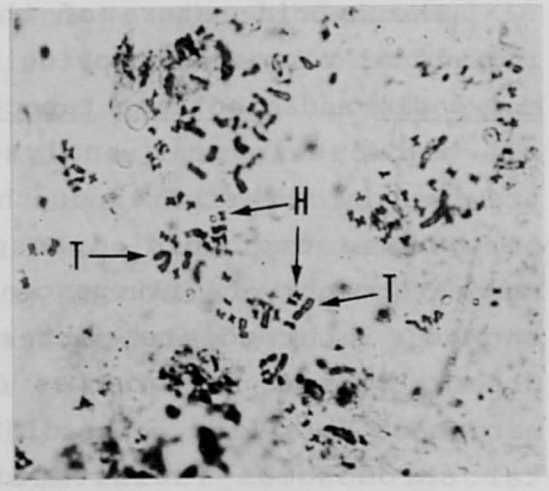


Fig. 4: Metaphase chromosomes of the hybrid line HT-1031. T = chromosomes of *Nicotiana tabacum* type, H = chromosomes of *Duboisia hopwoodii* type.

Cytological analyses of the cell line HT-1031 were conducted approximately 8 month after isolation. The small chromosomes of D. hopwoodii (2n=60) were distinguishable from the thicker and longer chromosomes of N. tabacum (2n=48). Metaphase plates of HT-1031 contained both type of chromosomes (Fig 4). Chromosome numbers varied from one plate to another and ranged from about 70 to about 240, indicating genetic instability in this line.

HT1031 callus was transferred to B5 medium containing 10^{-5} M BA and 3% sucrose on which both Duboisia and tobacco calluses produced shoots. Although the frequency of shoot regeneration was low, to date , we have been able to obtain 2 callus lines which produce adventitious shoots from HT1031 (Fig. 2-D). The shoot regeneration is described in detail in the next section.

Discussion

The hybrid nature of the HT-1031 line, obtained by individual cloning following protoplast fusion of Duboisia hopwoodii and Nicotiana tabacum, was confirmed by biochemical and cytological analyses. Indirect evidence was provided by the studies of the electrophoretic patterns of peroxidase and acid phosphatase, and the thin layer chromatography of fluorescent compounds. The HT-1031 line showed intermediate patterns of both parental species. Observations of chromosomes directly indicated the hybrid nature of this line. The differences in size of the parental chromosomes facilitated the identification of the hybrid.

Variation in the chromosome number observed in the hybrid cells suggests that asymmetric mitosis and endoreduplication take place together in the cell population.

This chromosomal variation can produce genetically heterogeneous cell population, which may facilitate the selection for the specific cells with desirable characteristics. Chromosomal instabilities have also been reported in the somatic hybrid cell lines of Atropa belladonna + Nicotiana chinensis (Gleba et al., 1982) and the hybrids Datura innoxia + Atropa belladonna (Krumbiegel and Schieder, 1979), which indicates that this phenomenon is prevalent among intergeneric hybrid cell lines of the Solanaceae. The heterogeneous shoot morphology and the abnormal growth of the shoots is also suggestive of genetic instability.

The effectiveness of the method for single cell culture used in this study still remains unclear, because we obtained only 1 colony out of 280 heterokaryons inoculated. This might have resulted partly from the low colony-regeneration rate of D. hopwoodii protoplasts. Plating efficiency of D. hopwoodii protoplasts when plated in normal agar medium was less than 0.15%. But in case of the hybrids of D. leichhardtii and N. tabacum described in the next section, rapid cell divisions ocuured even though D. leichhardtii protoplast did not show normal cell divisions. This method for single cell culture seems to be effective, when viability of the nurse cells is high and the process of cell fusion does not damage the protoplasts.

The low regeneration ability in the hybrid HT 1031 may be resulted from the low regeneration rate of the D. hopwoodii suspension culture. The cell suspension culture of D. hopwoodii used in this hybridization study had been cultured for about 2 years and did not regenerate shoots. Young calluses or cell suspension culture may be better materials than old cells for studies of somatic hybridization when plant regeneration from the heterokaryons is required. Altanatively, cells from intact plants such as

mesophylls, which have morphological totipotency, are a good material.

SECTION 2. Genetic Diversity Originating from a Single Hybrid Cell of *Duboisia hopwoodii* + *Nicotiana tabacum*

Parasexual hybridization by protoplast fusion has made it possible to produce hybrid plants between species belonging to different taxonomic tribes of the Solanaceae (Krumbiegel and Schieder 1979, 1981; Gleba et al. 1982, 1983; Skarzhynskaya 1982; Potrykus et al. 1984), of the Umbelliferae (Dudits et al. 1979, 1980), and of the Cruciferae (Gleba and Hoffmann 1978, 1979, 1980). Somatic hybridization between taxonomically remote species normally produces genetic instability (Gleba and Sytnik, 1984).

Hoffmann and Adachi (1981) made a thorough study of the genetic diversity in somatic hybrids derived from a single fusion cell of *Arabidopsis thaliana* + *Brassica campestris*. They obtained a large number of regenerants from a hybrid cell line and demonstrated the morphological and chromosomal heterogeneity of those plants. Hybrid plants formed between *Lycopersicon esculentum* and *Solanum rickii* also showed heterogeneous leaf shapes and ploidy even though they were offspring of a single fusion product (O'Connell and Hanson, 1986). Although the number of examples of intergeneric and interfamilial somatic hybridizations have increased, processes of changes in their genetic makeup during subculture are largely unknown.

A preliminary investigation conducted about 1 year after fusion showed that a hybrid cell line, HT 1031, contained cells that had diverse chromosome numbers. Here we describe in detail the genetic instability in this hybrid cell line after 3 years of culture and discuss the mechanisms involved in genetic fluctuation in somatic hybrids.

MATERIALS AND METHODS

Materials Approximately 1 year after the isolation of the hybrid cell line, HT1031, I divided its callus into approx. 500 subclones in the form of small cell aggregates, that have been maintained in light on solidified B5 media containing $2x10^{-5}$ M 1-naphthaleneacetic acid and $2x10^{-6}$ M 6-benzyladenine. Ten subclones that showed good growth were used in the investigation described here. For shoot regeneration, calluses were placed on B5 media with 10^{-5} M 6-benzyladenine.

Nuclear DNA content The nuclear DNA content was determined by microfluorometric analysis, the fluorescence emitted from the DNA-DAPI complex formed in the nuclei of protoplasts that had been fixed on a slide glass being measured. An enzyme solution containing 4% Cellulase Onozuka RS, 2% Macerozyme R-10 (both from Kinki Yakult, Co. Nishinomiya, Japan), 4% Driserase (Kyowa Hakko Co. Tokyo, Japan), 0.1% Pectlyase Y-23 (Seishin Pharmaceutical Co. Tokyo, Japan), 20 mM MES, 5mM $MgCl_2$, and 0.6 M sorbitol (pH 5.6) was used to obtain protoplasts from calluses. Two percent Cellulase Onozuka RS and 0.5% Macerozyme R-10 were used to isolate mesophyll protoplasts. The isolated protoplasts were washed twice with a solution containing 20 mM MES, 5 mM $MgCl_2$ and 0.6 M sorbitol (pH 5.6), after which 50 µl of the protoplast solution was placed on a slide glass then dehydrated and fixed in acetic acid:ethanol (1:3) for 30 min. DNA was stained overnight at 4°C with a solution containing 50 µg 4',6-diamino-2-phenylindole dihydrochloride (DAPI), 10 mM EDTA-2Na, 10 mM 2-mercaptoethylamine hydrochloride and 100 mM NaCl in 1000 ml Tris/HCl buffer (10 mM, pH 7.4) (Hamada and Fujita, 1983). Fluorescence was monitored and measured under an

epifluorescent microfluorometer (Olympus MMSP) equipped with a 365 nm interference excitation filter, a 400 nm dichroic mirror, a 420 nm cut filter, a 450 nm interference band pass filter, an objective lens (100/1.30) and a high pressure mercury lamp (200 W). The content of the nuclear DNA was expressed relative to that of tobacco mesophyll cells (2c=20.0).

Chromosome observation Calluses were treated with 0.025% colchicine for 2 h. They were fixed in acetic acid:ethanol (1:3) for 12 h at 4 ° C then stained with basic fuchsin.

Peroxidase analysis Cell-free extracts were prepared by grinding cells in cold buffer (50 mM Tris/HCl, pH 7.4) in a chilled mortar then centrifuging the slurry (12,000xg, 20 min). Isoelectric focusing was done for 3 h on rod gels containing 5% polyacrylamide, 2% Ampholine (pH 3.5-10, LKB) at constant voltage of 200 V. Deaminobenzidine tetrahydrochloride was used as the substrate in the activity staining of peroxidases.

RESULTS

Morphology Subclones from the hybrid cell line HT 1031 originally showed heterogeneous morphologies when first isolated from HT 1031 as small cell aggregates. The color of the calluses varied from pale yellow to bright green, and most were friable. Some had a very hairy appearance, not present in either parent species, because of elongated cells; the others were compact and hard. A common feature of all the subclones was necrosis of the top of the callus 2 to 3 weeks after subculture; in addition purple spots ap-

peared frequently on the surface (also seen on Duboisia callus).

During long-term maintenance in undifferentiated growth, most subclones came to have a pale green homogeneous appearance and were friable. One subclone (here designated subclone H) accumulated an extensive amount of purple pigment; but, the other subclones seemed to lose the ability to produce this pigment. Attempts to induce shoots from the subclones were unsuccessful until 2 years after fusion, when shoots regenerated from 2 subclones. These shoots, designated shoot culture 1 and 2, were maintained on regeneration media containing 10^{-5} M benzyladenine. The morphologies of these shoots are shown in Fig. 1. One had leaves similar to those of Duboisia,

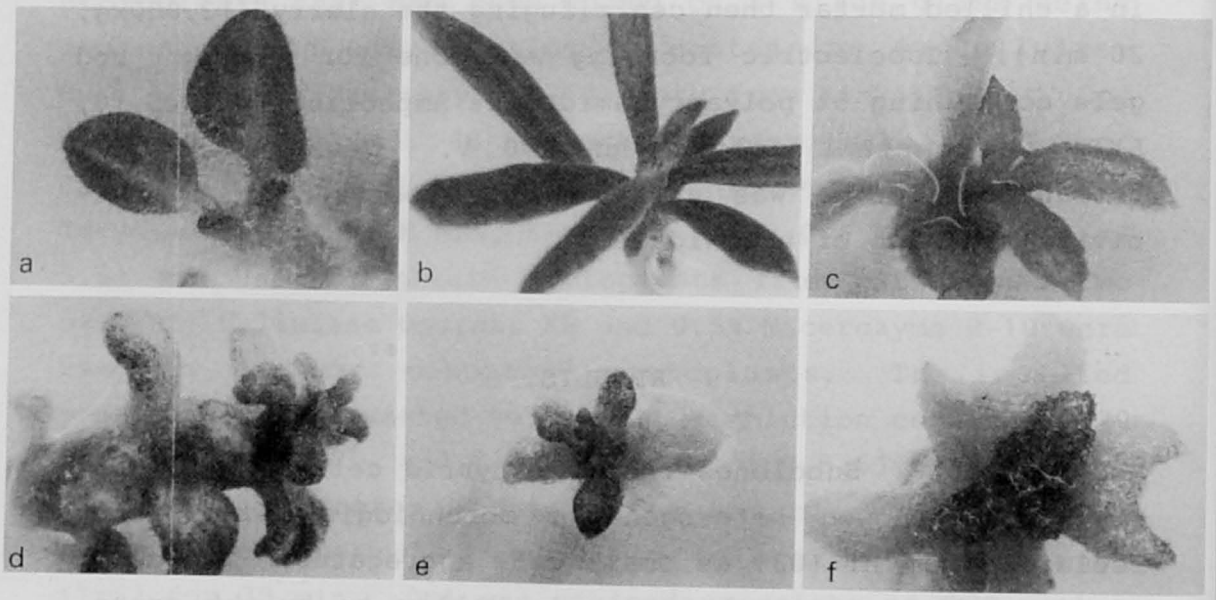


Fig. 1 Shoot morphologies of Nicotiana tabacum (a), Duboisia hopwoodii (b) and their hybrids (c, shoot culture 1; d-f, shoot culture 2).

narrow and pointed (shoot culture 1, Fig. 1 c); the other had broad tobacco-like leaves (shoot culture 2, Fig. 1 d-f).

When these shoots were treated for 3 days with a high concentration of 3-indolebutyric acid (10^{-4} M) then cultured in hormone-free media, adventitious roots formed at a high frequency. The growth of those plantlets did not, however, go beyond root initiation.

Nuclear DNA content Distributions of the relative nuclear DNA content of Nicotiana tabacum, Duboisia hopwoodii and the hybrid (HT 1031) were investigated approximately 1 year after fusion (Table 1). At that time, the HT 1031 line showed patterns intermediate between those of the parent species for peroxidase and acid phosphatase isozymes and for species-specific fluorescent compounds (see section 1). Calluses of N. tabacum and D. hopwoodii had 4c nuclear DNA contents, which suggests that the culture conditions I used favor the doubling of the nuclear

Table 1. Nuclear DNA contents of Nicotiana tabacum, Duboisia hopwoodii and their hybrid (HT 1031)

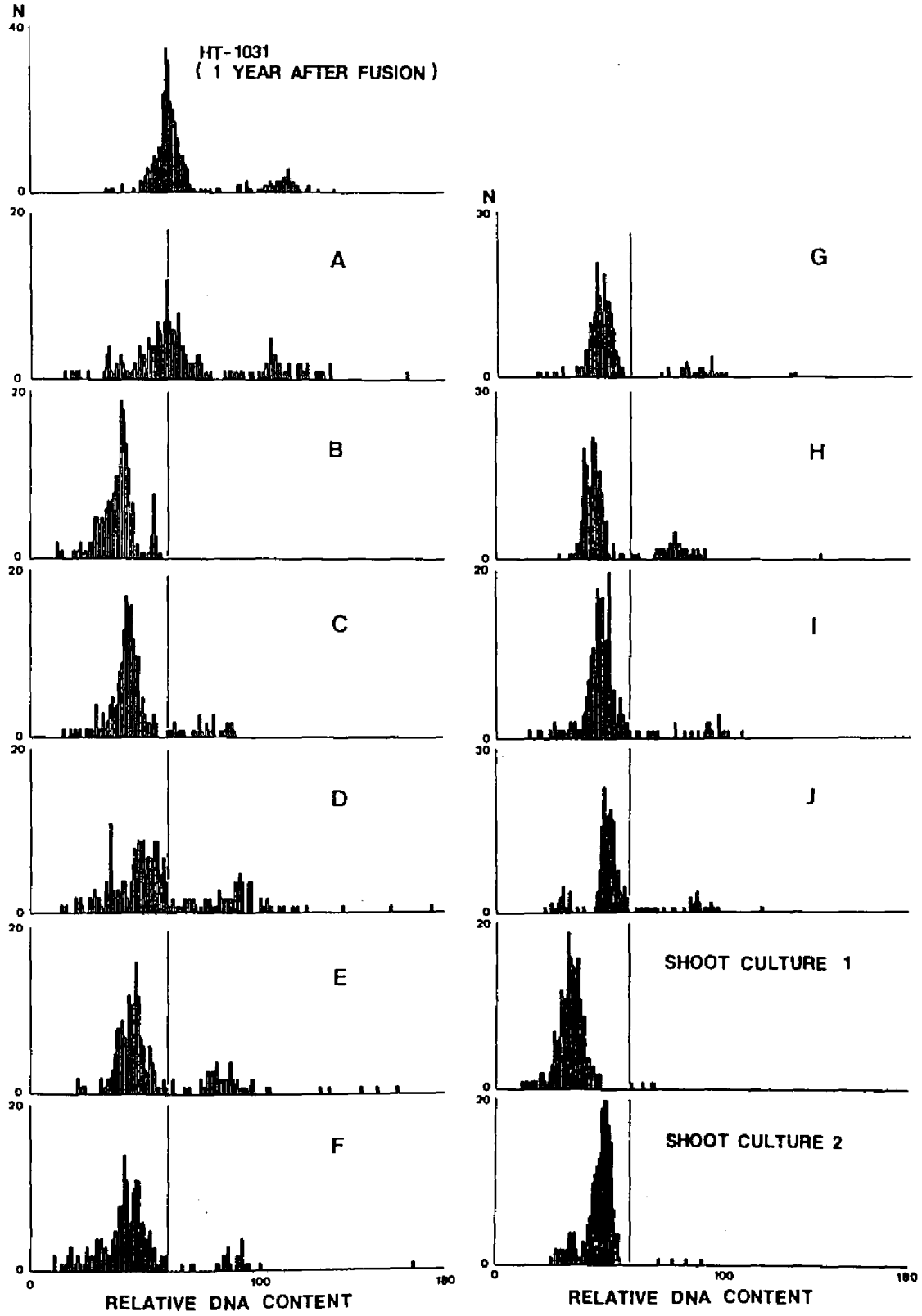
			Mean[1)]	SD[1,2)]	Frequency in total population (%)	Level of DNA content
N. tabacum						
Mesophyll	Peak	1	20.0	2.20	96.0	2c
		2	37.4	2.85	4.0	4c
Callus	Peak	1	36.5	4.18	63.1	4c
		2	69.0	4.71	33.0	8c
		3	134.7	20.56	3.9	16c
D. hopwoodii						
Mesophyll	Peak	1	7.7	0.64	98.4	2c
		2	14.6	1.94	1.6	4c
Callus	Peak	1	15.2	1.63	85.2	4c
		2	29.7	3.11	14.2	8c
		3	60.9	2.18	0.6	16c
HT 1031						
Callus	Peak	1	59.3	4.55	76.9	
		2	107.3	8.81	17.0	

1) Values expressed are relative to the nuclear DNA content of Nicotiana tabacum mesophyll cells (2c=20.0).
2) SD = standard deviation

DNA contents. The mean value for the main peak of the DNA distribution in the HT 1031 line which was a fusion product between N. tabacum (mesophyll) and D. hopwoodii (callus), was much larger than the sum of the 2c value for N. tabacum (mesophyll) and the 4c value for D. hopwoodii (callus), indicating that this hybrid is a product of multiple fusion. This high DNA content, however, might have been the result of somatic polyploidization after fusion. Although the distribution of the nuclear DNA content of the HT 1031 line showed two distinct peaks (Fig 2), cytological observations revealed heterogeneity in its chromosome number (see section 1). In addition, multinuclear cells were frequently present in this hybrid.

When the nuclear DNA contents of some of the subclones derived from the HT 1031 line were investigated approximately 3 years after fusion (Fig 2), all except subclone A had smaller DNA contents than the parent HT 1031 line 1 year after fusion. Subclones A and D had very broad distribution of their nuclear DNA contents in comparison to the other subclones. Multinuclear cells also were common in all the subclones. A cell containing 22 or 23 nuclei was found in subclone H. Most nuclei in a given multinuclear cell had the same DNA content level. The two shoot cultures derived from the HT 1031 line had different

Fig. 2 Distribution of the nuclear DNA contents in the HT 1031 line. The DNA content of the original line one year after fusion is compared with the DNA contents of subclones A-J and shoot cultures three years after fusion. The vertical line indicates the mean value of the main peak of the original HT 1031 (one year after fusion).


N
40
HT-1031
(1 YEAR AFTER FUSION)
0
20
A
B
C
D
E
F
100
180
RELATIVE DNA CONTENT
N
30
G
H
I
J
SHOOT CULTURE 1
SHOOT CULTURE 2
100
180
RELATIVE DNA CONTENT

nuclear DNA contents. Shoot culture 1 had a much smaller nuclear DNA content than shoot culture 2 and subclones maintained in unorganized growth.

Chromosome constitution The metaphase chromosomes of some subclones approximately 3 years after fusion are shown in Fig 3. Elimination of the Nicotiana type (long)

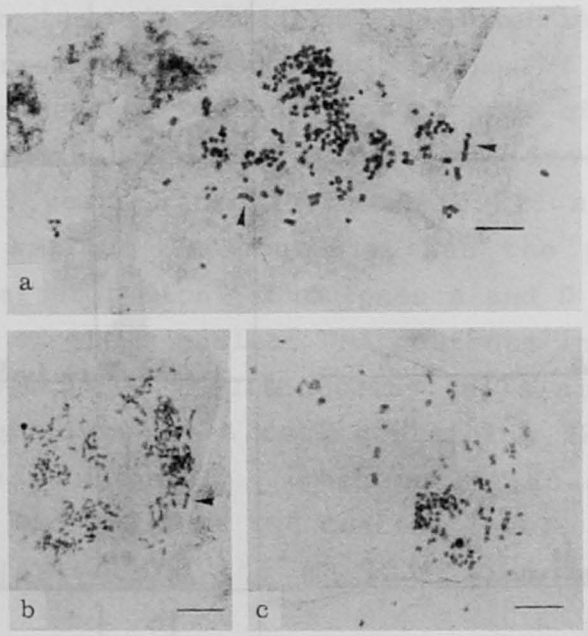


Fig. 3 Mitotic metaphase chromosomes of the hybrid cells Nicotiana tabacum + Duboisia hopwoodii . Arrows indicate a tobacco type chromosome. Bar: 10 µm. (a) A cell of subclone B, having 150-160 chromosomes; (b) and (c) Cells of subclone E, both having 120-130 chromosomes. In plate (b) some tobacco type chromosomes are present, whereas in plate (c) no such chromosomes are found.

chromosomes had taken place in all 10 subclones examined. The preliminary investigation conducted approximately 1 year after fusion had indicated no tendency for species-specific elimination. No Nicotiana type chromosomes were found in subclones C and J. Other lines had fewer than 20 Nicotiana type chromosomes per metaphase. Subclones E and H were chimeric in their chromosome constitutions; some metaphase plates contained only Duboisia type chromosomes, others contained mixtures of Nicotiana and Duboisia type chromosomes. In most of the metaphase plates observed, the chromosome numbers ranged from 100 to 170.

Peroxidase Isoelectric focusing of the peroxidase from the HT1031-derived subclones was done 3 years after fusion. Activity staining of peroxidase with diaminobenzidine showed species-specific peroxidases with acidic pIs which provided marker bands (arrows, Fig 4). Only subclones E, F and H showed the Nicotiana-specific band; the other lines all showed Duboisia-like peroxidase patterns, a

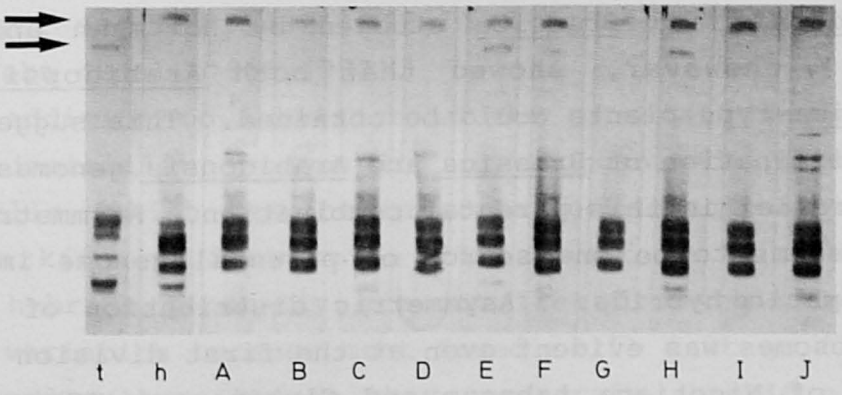


Fig. 4 Peroxidase isozymes from Duboisia hopwoodii (h), Nicotiana tabacum (t) and hybrid subclones (A-J). Arrows indicate species-specific bands.

reflection of the loss of the Nicotiana genes in these hybrid calluses.

DISCUSSION

Ten subclones derived from the intertribal hybrid cell line Duboisia hopwoodii + Nicotiana tabacum showed considerable genetic instability. Microscopic observations of chromosomes and peroxidase analysis provided evidence that the Nicotiana genome was eliminated. The decrease found in the DNA content is partly attributable to the elimination of the Nicotiana genome. Whether the Nicotiana chromosomes that remained in some subclones eventually will be eliminated will be determined with further monitoring of this hybrid.

Although elimination of the chromosomes of one parent is common in intergeneric and interfamilial hybrids, the mechanism for this elimination is not clear. In most cases, species-specific elimination takes place (Kao 1977, Krumbiegel and Schieder 1981, Gleba et al. 1983, Potrykus et al. 1984, Tabaeizadeh et al. 1985). The hybrids of Arabidopsis and Brassica studied by Hoffmann and Adachi (1981), however, showed that both Arabidopsis- and Brassica-type plants could be obtained. This suggests that the elimination of Brassica and Arabidopsis genomes occurs in parallel in this parental combination. Asymmetric division seems to be one source of parental genome imbalances in somatic hybrids. Asymmetric distribution of parental chromosomes was evident even at the first division of fused cells of Nicotiana tabacum and Glycine max (Chien et al. 1982). This phenomenon may produce a genetically heterogeneous cell population and, during subculture, preferential selection of a certain cell type may take

place. Another possible explanation for species-specific genome elimination is preferential chromosome elimination from a homogeneous cell population by an unknown mechanism. In the case of Duboisia hopwoodii + Nicotiana tabacum, it is notable that the elimination of the tobacco chromosome seemed to occur slowly. Elimination was not evident during the early stage of culture, only being manifested 3 years after fusion.

In the HT 1031 hybrid line, attempts to regenerate plants during the early stage of culture were unsuccessful, no regenerants being obtained until 2 years after fusion. This indicates that some form of genetic incompatibility may have existed during the early stage of culture which prevented the hybrid cells from regenerating plants. After the elimination of tobacco chromosomes, such incompatibility might be lessened or disappear. A similar observation has been reported for Datura innoxia + Atropa belladonna somatic hybrids (Krumbiegel and Schieder, 1981). Genetic instability in interfamilial and intergeneric hybrids often results in hybrid cells becoming incapable of morphogenesis or in the formation of abnormal plants that frequently lack root organogenesis (Dudits et al. 1979; Krumbiegel and Schieder 1979, 1981; Gleba and Hoffmann 1980; Gleba et al. 1982,1983).

In addition to imbalance in the parental genome, the genetic instability common to any unorganized culture of plant cells, which results in so-called somaclonal variation (Larkin and Scowcroft 1981), must be considered. Somatic hybrids generally are not free from this type of genetic variation because most fused cells must go through unorganized culture before they regenerate hybrid plants. For example, multinuclear cells, common in calluses and suspension-cultured cells, were frequently present in HT 1031 calluses.

In conclusion, individual somatic hybrids, even from a single fusion product, should be considered unique genetic material which may serve to improve existing plant species and to develop novel plants.

SECTION 3. Alkaloid Biosynthesis in Somatic Hybrids, *D. leichhardtii* + *N. tabacum*.

In the previous sections, I have demonstrated that a somatic hybrid cell line obtained by protoplast fusion of *Duboisia hopwoodii* and *Nicotiana tabacum*, had morphogenetic ability although it showed considerable genetic instability. In this section, I describe fusions of mesophyll protoplasts of *N. tabacum* and protoplasts from cell suspension culture of *D. leichhardtii* which was derived from a root culture producing large amounts of tropane alkaloids (see section 2 of the previous chapter). Recently developed electrostimulation method is employed for protoplast fusion (Bates et al. 1985, Yamada and Morikawa 1985, Morikawa et al 1986, Zachrisson and Bornman 1986). Alkaloid biosynthesis of the somatic hybrids is studied.

Materials and methods

Plant materials A line of cell suspension culture was established from a root culture of *Duboisia leichhardtii* which produced considerable amounts of scopolamine, hyoscyamine and nicotine. This cell suspension culture has been maintained in the dark in B5 medium with $2x10^{-5}$ M NAA and $2x10^{-6}$ M BA. The methods for protoplast isolation from this cell suspension and from leaves of *Nicotiana tabacum* c.v. Samsun was the same as in section 1.

Protoplast fusion Protoplasts of *D. leichhardtii* were fused with mesophyll protoplasts of *N. tabacum* by electric stimulation. The method of electrofusion was essentially that of Yang and Yamada (1988). After the pearl chain formation with alternating current of 100 V_{p-p}/cm at 500 kHz

for 60 sec, fusion was induced with a direct current pulse of 1 kV for 40 usec in an SSH-1 Somatic Hybridizer (Shimadzu Corporation, Kyoto, Japan). A fusion medium containing 0.5 M glucose and 2.5 mM $CaCl_2$ was used during this procedure.

Culture of heterokaryons After fusion treatment, protoplasts were cultured for 2 to 4 days in Nagata and Takebe medium (1971) supplemented with 5% coconut milk. Hybrid cells were distinguishable from parental protoplasts because they contained both chloroplasts from tobacco mesophylls and dense cytoplasmic strands from Duboisia suspension cells as shown in section 1. Heterokaryons were picked up with a micropipette and individually cultured in a nylon mesh chamber surrounded by nurse cells. The cell colonies formed were cultured on B5 agar medium with $2x10^{-5}$ M NAA and $2x10^{-6}$ M BA. Shoots were regenerated and maintained on B5 agar medium with $2x10^{-6}$ M BA.

Cytological investigation Cells were fixed in acetic acid:ethanol (1:3) then treated in 5% macerozyme at 37° C for 30 min before staining with acetorcein.

Ribosomal RNA genes (rDNA) analysis Total DNA was extracted from shoot cultures and digested with restriction endonucleases, Bam HI and Dra I, sequentially. The digested DNA was applied to a 0.8% agarose gel, and electrophoresis was done for 5 hr at 70 V. A Southern blot hybridization (Southern 1975) was conducted with ^{32}P-labelled rRNA from rice cells as the probe (Uchimiya et al. 1983). Gene Screen Plus (NEN Research Products) was used as hybridization-transfer membrane. Rice rRNA was extracted from callus tissues (Oryza sativa, c.v. Fujimionori) after Oono and Sugiura (1980). A mixture of purified 25S and 17S rRNAs was

labeled with ^{32}P using T4 polynucleotide kinase and [^{32}P]-ATP.

Precursor feeding Each alkaloid precursor was fed to 1g of shoot culture in 10 ml of liquid B5 medium containing $2x10^{-6}$ M BA and incubated for 7 days. Alkaloids were extracted as described in the previous chapter. For extraction of hydrophilic alkaloids such as hygrine and tropine, a mixture of CH_2Cl_2:propanol (15:85) was used in the phase partitioning step (Hartmann et al. 1986). The method for alkaloid quantification with gas-liquid chromatography was the same as described in the chapter I except that a lower column temperature (100-260°C) was used.

Hyoscyamine 6β-hydroxylase activity Preparation of cell-free extract and measurement of the enzyme activity was conducted after Hashimoto et al. (1986).

RESULTS

Isolation and culture of heterokaryons Protoplasts from the D. leichhardtii cell suspension culture began dividing 36 hr after fusion treatments, but they stopped growing after the first or second division. Tobacco protoplasts first divided 2 or 3 days after fusion. Heterokaryons which was distinguishable from parental cells on the basis of the presence of both chloroplasts from tobacco mesophylls and dense cytoplasm from Duboisia suspension cells divided as early as the Duboisia protoplasts: by day 7 they had developed into small cell aggregates, while most tobacco cells remained at the first or second cell division. This fast growth of the heterokaryons facilitated the visual selection. They were

picked out and individually transferred to the nurse culture system at day 2-7. The heterokaryons transferred at early stages of development died, but those transferred at day 5-7 developed into cell colonies in the nylon mesh chambers. After 1 month incubation in the nurse cultures, 6 putative hybrid colonies were transferred to B5 agar medium. All the 6 cell lines showed shoot regeneration upon transferring to the regeneration medium (see MATERIALS AND METHODS section). The regenerated shoots showed considerable variety in morphology. Hybrids C and D were similar to Nicotiana, while hybrid A was like Duboisia. Hybrid F had intermediate morphology and hybrids B and E developed only abnormal shoots.

Genetic analysis The small chromosomes of Duboisia (2n=60) were distinguishable from the longer chromosomes of Nicotiana (2n=48) (previous section). Cytological analysis

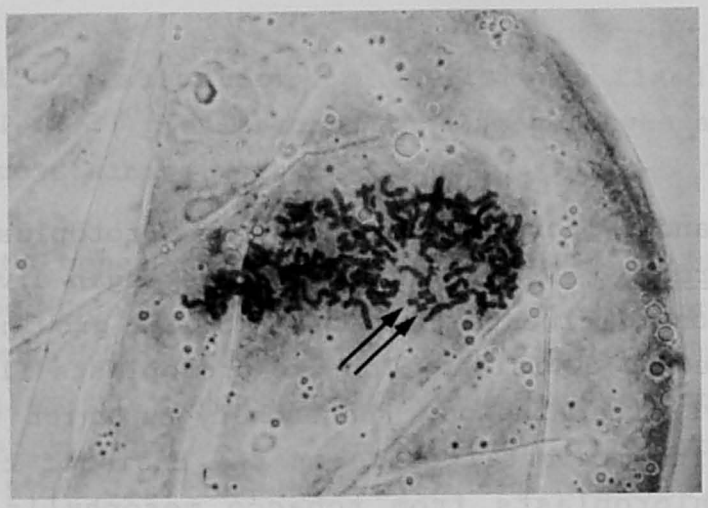


Fig. 1. A metaphase plate from hybrid cells of Duboisia leichhardtii and Nicotiana tabacum. Arrows indicate Duboisia-type chromosomes.

of the putative hybrid cells showed that metaphase plates from all 6 cell lines contained both types of chromosomes (Fig. 1). Chromosome numbers varied from one plate to another: most cells contained 120 to 150 chromosomes.

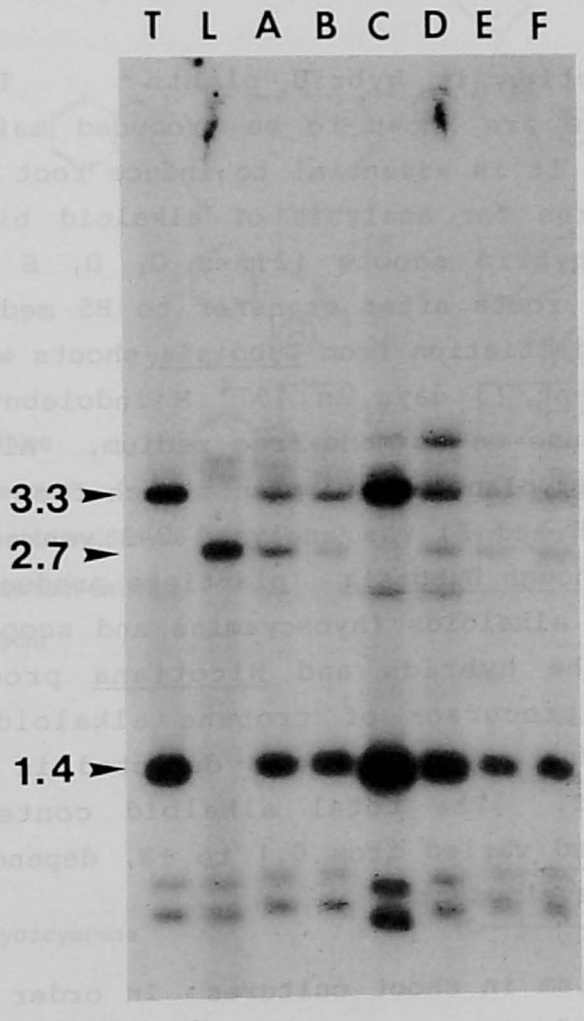


Fig. 2. Autoradiographs of the Southern blot hybridization of ^{32}P-rRNA from rice and digested (Bam HI + Dra I) DNA from shoot cultures of Nicotiana tabacum (T), Duboisia leichhardtii (L) and the hybrids (A-F).

The nuclear genome composition of the shoots induced from the hybrid cells was analyzed by a Southern blot hybridization with rice rRNA (Fig. 2). All 6 shoot cultures showed Duboisia- and Nicotiana-specific restriction fragments. Line C had a very weak Duboisia-specific band (2.7 kb), indicating that the Duboisia nuclear genome was mostly eliminated in this shoot culture.

Alkaloid production in hybrid plants Tropane and nicotine alkaloid are known to be produced mainly in the roots; therefore it is essential to induce root organs from the shoot cultures for analysis of alkaloid biosynthesis. Four lines of hybrid shoots (lines C, D, E and F) and Nicotiana formed roots after transfer to B5 medium without hormones. Root initiation from Duboisia shoots was achieved by auxin treatment (3 days in 10^{-4} M indolebutylic acid) followed by culture on hormone-free medium. Alkaloid composition in hybrid plantlets (3 plants each from lines C and D, 1 each from E and F) was analyzed 2-3 weeks after root initiation. Although Duboisia plantlets produced the same level of tropane alkaloids (hyoscyamine and scopolamine) as of nicotine, the hybrids and Nicotiana produced only nicotine. No precursor of tropane alkaloids such as hygrine, tropinone or tropine was detected in the hybrid plants, either. The total alkaloid content of the plantlets analyzed varied from 0.1 to 1%, depending on the degree of root development.

Alkaloid metabolism in shoot cultures In order to confirm that the hybrid plants retained the Duboisia genes involved in tropane alkaloid biosynthesis, we examined all reaction steps in alkaloid synthesis, from N-methylputrescine to scopolamine and to nornicotine (Fig. 3, steps a to j), by feeding each alkaloid precursor to the shoot culture and

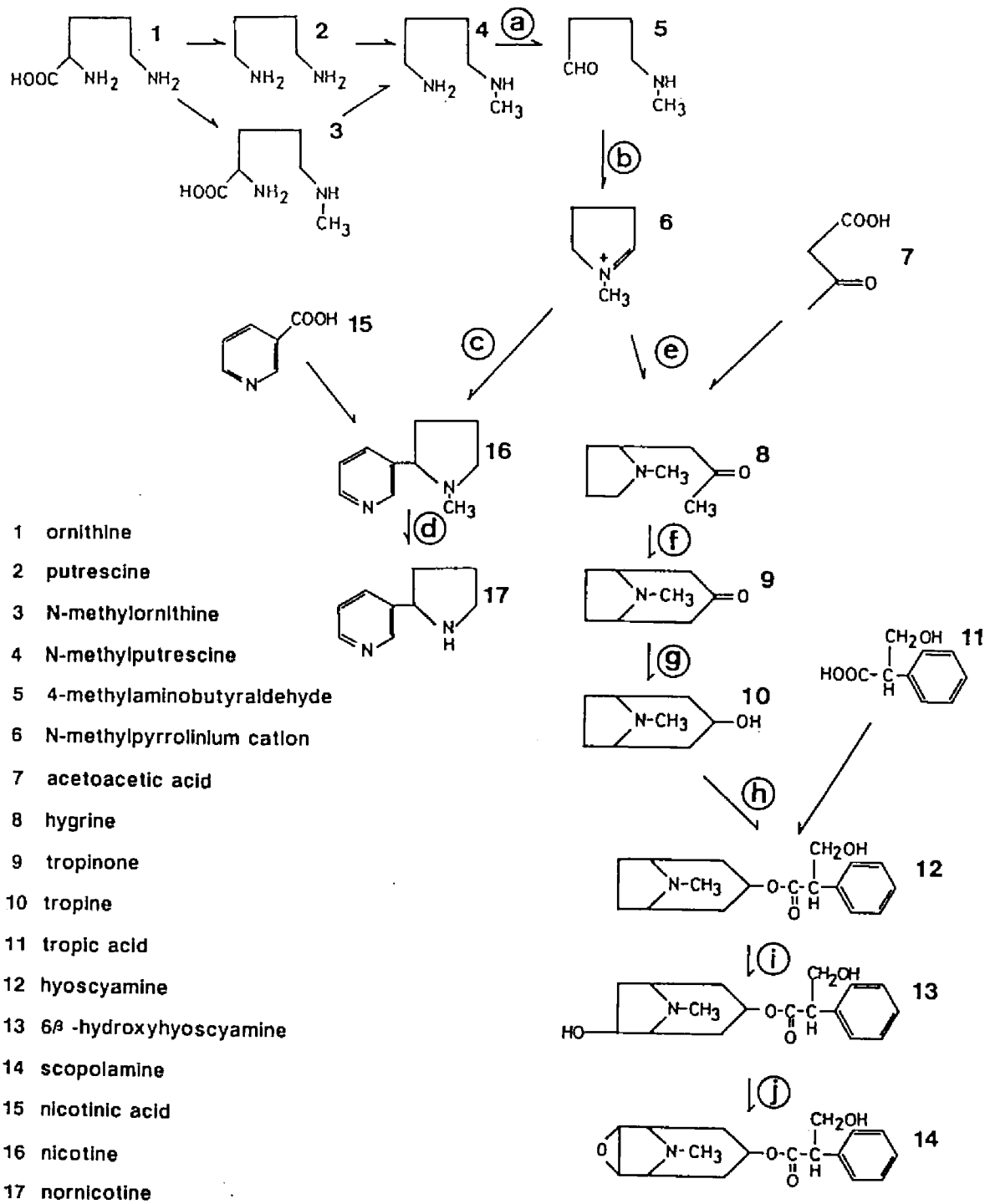


Fig. 3 Biosynthetic pathway of tropane and nicotine alkaloids

analyzing the direct product of the reaction step. I used the shoot cultures as experimental material despite their low alkaloid production because 1) it was difficult to obtain a large number of homogeneous plantlets, and 2) the shoot cultures expressed most of the biosynthetic activities for alkaloids production, as shown below. First, I examined the biosynthetic activity in both parental species. Then the activity in the hybrids was analyzed where parental species responded differently upon precursor feeding.

a) N-methylputrescine → 4-methylaminobutyraldehyde (MABA)

To analyze MABA in gas-liquid chromatography, MABA was chemically coupled with acetoacetic acid (pH 9 at 60°C for 30 min), after which the hygrine formed was quantified. In advance I had confirmed that no endogenous MABA or hygrine was present in any shoot culture (Table 1). Upon feeding of

Table 1

Methylputrescine → 4-Methylaminobutyraldehyde (MABA)

	methylputrescine added (µmol/flask)	MABA formed (µmol/flask)
Duboisia	0	nd
Nicotiana	0	nd
Duboisia	10	1.28
Nicotiana	10	2.34

MABA was chemically coupled with acetoacetic acid after which hygrine formed was quantified by gas chromatography.

N-methylputrescine, MABA was detected in both Duboisia and Nicotiana shoots.

b) **MABA → N-methylpyrrolinium ion** MABA exists in equilibrium with N-methylpyrrolinium ion in physiological conditions.

c) **MABA + nicotinic acid → nicotine** Small amounts of endogenous nicotine were detected in Nicotiana and hybrids C and D (Table 2). The biosynthetic activity in this step was determined by subtracting the amount of endogenous nicotine from the amount of nicotine after feeding with MABA and nicotinic acid. The activity was found in Nicotiana and all 6 hybrid shoots. Duboisia, whose roots produce a considerable amount of nicotine, did not show this coupling activity in the shoot culture.

Table 2

MABA (Methlaminobutyraldehyde) + Nicotinic acid ⟶ Nicotine

	Nicotine endogenous (µmol/flask)	Nicotine after feeding (µmol/flask)	Nicotine converted (µmol/flask)
Duboisia	nd	nd	nd
Nicotiana	0.10	0.29	0.19
A	nd	0.20	0.20
B	nd	0.03	0.03
C	0.07	0.10	0.03
D	0.07	0.17	0.10
E	nd	0.01	0.01
F	nd	0.04	0.04

Nicotine converted = Nicotine after feeding - Nicotine endogenous.

Table 3

Nicotine ⟶ Nornicotine

	nicotine (μmol/flask)		nornicotine (μmol/flask)		recovery (%)
	shoot	medium	shoot	medium	
Duboisia	0.57	0.18	0.30	nd	9.9
Nicotiana	4.21	0.59	nd	nd	47.7
A	3.67	0.21	+	nd	38.8
B	5.11	nd	0.05	nd	51.6
C	5.27	0.36	nd	nd	56.2
D	6.13	nd	+	nd	61.3
E	4.77	0.18	nd	nd	49.4
F	5.29	nd	0.05	nd	53.3

d) nicotine → nornicotine This demethylation activity was detected in Duboisia (Table 3) but not in Nicotiana, although Nicotiana genetically has this activity, which is expressed in curing leaves. The activity in the hybrid shoots was low. The recovery of nicotine + nornicotine in the hybrids and Nicotiana was approximately 50% while Duboisia shoots degraded 90% of the nicotine fed in 7 days.

e) MABA + acetoacetic acid → hygrine The precursorship of acetoacetic acid has not been fully demonstrated. MABA and acetoacetic acid are coupled to form hygrine in physiological pH and temperature without any enzymes (see section 3 in the previous chapter).

f) hygrine → tropinone The activity of this ring formation step was not detected in either Duboisia or Nicotiana shoot culture.

g) tropinone → tropine Duboisia and all hybrid shoots except line C efficiently converted tropinone to tropine

Table 4

Tropinone ⟶ Tropine

	tropinone (μmol/flask)	tropine (μmol/flask)
Duboisia	0.75	3.30
Nicotiana	3.03	nd
A	nd	6.33
B	0.07	5.18
C	5.10	nd
D	0.50	4.51
E	0.37	5.19
F	0.43	5.27

(Table 4). Nicotiana apparently did not have this activity, which is involved in tropane alkaloid synthesis.

h) tropine + tropic acid → hyoscyamine This esterification was not detected in either Duboisia or Nicotiana.

i) hyoscyamine → 6β-hydroxyhyoscyamine This activity was found in Duboisia and all hybrids except for line C, but not in Nicotiana (Table 5). Activity of hyoscyamine 6β-hydroxylase, which was recently isolated from Hyoscyamus niger and characterized by Hashimoto et al. (1986, 1987), was also examined (Table 6). Crude proteins from the hybrid shoots showed the hydroxylase activity, which was in accordance with the result of the precursor feeding.

j) 6β-hydroxyhyoscyamine → scopolamine The epoxidation activity was found in Duboisia and 5 hybrid shoots (Table 7). Nicotiana and Nicotiana-like hybrid C did not have this ac-

Table 5

Hyoscyamine ⟶ 6β-Hydroxyhyoscyamine

	hyoscyamine (μmol/flask)		6β-hydroxyhyoscyamine (μmol/flask)	
	shoot	medium	shoot	medium
Duboisia	3.49	2.24	0.25	nd
Nicotiana	5.42	nd	nd	nd
A	4.79	0.08	0.40	nd
B	5.13	0.19	0.23	nd
C	5.32	0.03	nd	nd
D	5.92	nd	0.02	nd
E	4.65	nd	0.18	nd
F	3.93	0.87	0.23	nd

Table 6

Hyoscyamine 6β-Hydroxylase Activity in Hybrid Shoots

	pkat/gFW cell	pkat/mg protein
Duboisia	1.47	2.62
Nicotiana	nd	nd
A	0.40	1.41
B	0.04	0.26
C	nd	nd
D	0.03	0.17
E	0.09	0.40
F	0.58	2.02
(Duboisia root culture)	23.6	15.2

tivity. Results of the feeding of alkaloid precursors to the hybrid shoots are summarized in Table 8.

Table 7

6β-Hydroxyhyoscyamine ⟶ Scopolamine

	6β-hydroxyhyoscyamine (μmol/flask)		scopolamine (μmol/flask)	
	shoot	medium	shoot	medium
Duboisia	4.17	0.64	0.63	nd
Nicotiana	5.67	0.20	nd	nd
A	5.47	nd	0.29	nd
B	4.77	nd	0.07	nd
C	6.21	0.04	nd	nd
D	6.33	0.37	0.05	nd
E	5.56	nd	0.09	nd
F	5.76	0.04	0.10	nd

Table 8 Alkaloid Biosynthesis in Hybrid Shoots

			hybrid					
	Duboisia	Nicotiana	A	B	C	D	E	F
nic endogenous	-	●	- D	- D	● N	● N	- D	- D
nic degradation	●	-	- N	- N	- N	- N	- N	- N
c) MABA+NA → nic	-	●	● N	● N	● N	● N	● N	● N
d) nic → nornic	●	-	● D	● D	- N	● D	- N	● D
g) tropinone → tropine	●	-	● D	● D	- N	● D	● D	● D
i) hyos → hyos-OH	●	-	● D	● D	- N	● D	● D	● D
j) hyos-OH → scop	●	-	● D	● D	- N	● D	● D	● D

nic = nicotine, MABA = 4-methylaminobutyraldehyde, NA = nicotinic acid
nornic = nornicotine, hyos = hyoscyamine, hyos-OH = 6β-hydroxyhyoscyamine
scop = scopolamine
● : activity positive

Biotransformation of hygrine in hybrid plantlets Hygrine was fed to plantlets from hybrids C, D and F 2 to 3 weeks after root initiation (Table 9). After a 1-week incubation hyoscyamine and scopolamine were detected in hybrid F, and tropine was found in hybrid D. The Nicotiana-type hybrid C did not biotransform hygrine into any tropane alkaloids.

Table 9 Incorporation of Hygrine into Tropane Alkaloids

	% incorporation		
	tropine	hyoscyamine	scopolamine
hybrid C	nd	nd	nd
D	0.09	nd	nd
F	nd	0.02	0.11

Discussion

Intergeneric hybrids of Duboisia leichhardtii and Nicotiana tabacum, obtained by individual cloning following electrofusion, produced nicotine but not any tropane alkaloids, although they contained the Duboisia nuclear genome involved in tropane alkaloid biosynthesis, which was demonstrated by a series of precursor feedings to the shoot cultures and plantlets of these hybrids.

The hybrid nature of the shoot cultures was confirmed by rRNA gene analysis. The Southern blot pattern of hybrid C indicated the elimination of the Duboisia nuclear genome, which was also shown by the precursor feeding experiments. Cytological analysis of callus tissues of this line indi-

cated no such elimination, suggesting that the elimination took place during shoot organogenesis. Contrary to this result, a somatic hybrid cell line of D. hopwoodii and N. tabacum showed elimination of Nicotiana chromosomes during long-term culture in unorganized growth (see section 2). Different types of selection pressure may work on genetically heterogeneous hybrid cells in unorganized growth and in the redifferentiation process.

The precursor feeding experiments showed that the shoot culture of Duboisia partly expressed alkaloid biosynthesis, even though its alkaloid production was completely repressed. Duboisia shoots showed no biotransformation activity of nicotine synthesis from MABA and nicotinic acid (step c), tropinone synthesis from hygrine (step f), or hyoscyamine formation from tropine and tropic acid (step h). It is noteworthy that Duboisia shoots convert nicotine to nornicotine because, to our knowledge, nornicotine has not been demonstrated in this species. It should be mentioned, however, that D. hopwoodii plants contained a large amount of nornicotine in the leaves (see section 2 of the previous chapter). The rapid degradation of nicotine found in Duboisia might make it difficult to isolate nornicotine in the leaves of this plant. Also this rapid degradation may explain why nicotine is not accumulated in the leaves of D. leichhardtii (nor in D. myoporoides), even though its roots produce a large amount of nicotine as well as tropane alkaloids. Nicotiana shoots produced nicotine, but the level of production was much lower than that in the roots.

The lack of tropane alkaloids in the shoot cultures facilitated the detection and identification of small amounts of alkaloids formed upon precursor feeding. In general the hybrid shoots except for hybrid C showed both activities specific in Nicotiana (nicotine formation from MABA and nicotinic acid, step c) and in Duboisia

(activities involved in tropane alkaloid synthesis and nornicotine synthesis, step d). But some activities such as endogenous nicotine production and nicotine degradation were not expressed "dominantly" in the hybrid shoots.

Feeding of hygrine to the young hybrid plants showed that hybrid F had activity to synthesize scopolamine from hygrine, and that hybrid D could synthesize tropine. This result indicated that the Duboisia genome was not eliminated during the process of root organization. The suppression of tropane alkaloid production may be the result of preferential distribution of N-methylpyrrolinium ion into nicotine synthesis at the fork in nicotine/tropane alkaloid biosynthesis. Recently nicotine synthase activity has been found in tobacco roots (Leete, personal communication) while hygrine seems to be formed non-enzymatically. In Duboisia a set of nicotine synthase genes may produce an amount of the enzyme which competes with non-enzymatic (or enzymatic) hygrine formation for N-methylpyrrolinium, which may result in the production of the same level of tropane and nicotine alkaloids. Duboisia + Nicotiana hybrids have one set of nicotine synthase gene from Duboisia and 2 sets from Nicotiana (N. tabacum is an amphidiploid), which may produce an excess amount of the enzyme sufficient to totally exclude hygrine formation. Or nicotine synthase from Nicotiana may have a much stronger affinity to N-methylpyrrolinium ion than that from Duboisia. Another possible explanation for the suppression of tropane alkaloid formation is an altered synthesis of acetoacetic acid. Acetoacetyl CoA, the activated form of acetoacetic acid, is an intermediate of terpenoid synthesis through the mevalonate pathway and degradation of fatty acids and some amino acids. In Nicotiana this compound may be metabolized rapidly rather than used for alkaloid synthesis, and in hybrids the tobacco-type metabolism might be dominantly expressed. It has been

demonstrated that in a root culture of N. tabacum, hygrine was formed by the coupling of exogenously applied acetoacetic acid and endogenously formed N-methylpyrrolinium ion (see section 3 in the previous chapter). This result and the lack of hygrine and tropane alkaloid synthesis in tobacco suggest that in N. tabacum plants, acetoacetic acid is not present in normal conditions.

In order to analyze quantitative expression of alkaloid synthesis, homogeneous and fast growing root cultures might be the ideal materials. But, to date, attempts to induce root culture from the hybrids have been unsuccessful.

Most hybrid plants showed abnormal morphology; only hybrid line C produced normal tobacco-like plants which flowered, although they were male sterile. The Duboisia nuclear genome may not be completely eliminated or the cytoplasmic genome may retain Duboisia genes, which may result in incompatibility in Nicotiana-dominated hybrid plants.

CONCLUSIONS

I have investigated alkaloid biosynthesis in cultured tissues of Duboisia and somatic hybrids of Duboisia and Nicotiana. My findings reported in the preceding chapters are summarized as follows:

Unorganized calluses of Duboisia have the genetic potential to produce tropane and nicotine alkaloids, but expression is repressed. When shoots are produced from unorganized calluses the ability to convert hyoscyamine to scopolamine appears, and upon root initiation the whole pathway of alkaloid biosynthesis is expressed.

Cultured roots of three species of Duboisia produced both tropane and nicotine alkaloids. Among the three species D. leichhardtii roots showed the best growth and the highest alkaloid contents. Cultured roots with high scopolamine content (1.16% on a dry weight basis) was selected.

Hygrine, an intermediate in biosynthesis of tropane alkaloids, was formed non-enzymatically by the coupling of 4-methylaminobutyraldehyde with either acetoacetic acid or acetonedicarboxylic acid. When these ketoacids were fed to a tropane alkaloid-free culture of tobacco roots, hygrine formation was observed. A possible process of this reaction was discussed.

Fusion of protoplasts from Nicotiana tabacum mesophyll cells and protoplasts from Duboisia hopwoodii suspension cultures was induced by polyethylene glycol treatment. Heterokaryons were isolated with a micropipette and were

cloned individually in small nylon mesh chambers, around which mesophyll protoplasts of tobacco were cultured as nurse cells. One putative intergeneric hybrid cell line was obtained, and the hybrid nature of this line was confirmed by isozyme analyses, analysis of fluorescent compounds and cytological investigations.

The genetic instability of the intertribal hybrid cell line, Duboisia hopwoodii + Nicotiana tabacum, was studied. Ten subclones of calluses derived from this hybrid cell line have been cultured for three years, and their genetic makeup has been clarified as to nuclear DNA content, chromosome constitution and peroxidase isozymes.

Nuclear DNA content differed in each subclone. For most subclones the mean DNA contents were smaller than the mean DNA content for the original hybrid cell line determined one year after fusion. This decrease in DNA content is partly attributable to the elimination of tobacco chromosomes which took place in all the subclones. The extent of the elimination of tobacco chromosomes varied among the subclones, evidence that chromosome elimination occurred slowly. The peroxidase isozyme analysis indicated the loss of a tobacco-specific isozyme, confirming the result of chromosome analysis.

Shoots regenerated from two hybrid subclones after two years also were heterogeneous in morphology and nuclear DNA content.

Somatic hybrids of D. leichhardtii and N. tabacum were obtained with individual cloning following electrofusion. The hybrid nature of the cloned cells and regenerated shoots was confirmed by cytological investigation and rDNA analysis, respectively. The hybrid plantlets produced only nicotine but not any tropane alkaloids, while Duboisia

plantlets produced both tropane and nicotine alkaloids. Presence of Duboisia genes involved in tropane alkaloid biosynthesis in the hybrid plants was demonstrated with a series of precursor-feeding experiments. Alkaloid metabolism in shoot cultures of Duboisia and Nicotiana, and mechanism of suppression of tropane alkaloid production in the somatic hybrids were discussed.

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CHAPTER II

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